Modulatory effects of nitric oxide and juvenile hormone on the control of reproductive behavior in female *Chorthippus biguttulus*

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Herewith I declare that I prepared the PhD Thesis "Modulatory effects of nitric oxide and juvenile hormone on the control of reproductive behavior in female *Chorthippus biguttulus*" on my own and with no other sources and aids than quoted.

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List of abbreviations

ACTH	Adrenocoricotropic hormone
AG	Aminoguanidine hemisulfate
AL	Antennal lobe
aL	α lobe
ASTs	Allatostatins
BSA	Bovine serum albumine
bL	β lobe
CA	Corpus allatum/corpora allata
CB	Central body
CBl	CB lower division
CBu	CB upper division
CC	Corpus cardiacum/corpora cardiaca
CCAP	Crustacean cardioactive peptide
cGMP	Cyclic guanosine monophosphate
Dip-Ast-7	*Diploptera punctata* allatostatin 7
DPM	Disintegrations per minute
ER	Endoplasmatic reticulum
FSH	Follicle stimulating hormone
GA	Glutaraldehyde
GABA	Gamma-aminobutyric acid
GEE	Generalized estimating equations
GnIH	Gonadotropin inhibitory hormone
JH	Juvenile hormone
LC-MS	Liquid chromatography-mass spectrometry
LH	Luteinizing hormone
Mas-AT	*Manduca sexta* allatotropin
Mbd	Median bundle

mL	Medial lobe
MSHα	melanocyte stimulating hormone
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NADPHd	NADPH diaphorase
NBT	Nitroblue tetrazolium
NCA	Nervus corporis allati
NCC	Nervus corporis cardiaci
NCS	Normal donkey serum
NGS	Normal goat serum
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
P	Pedunculus
PBS	Phosphate buffer saline
PBST	PBS with Triton-X-100
PCi	Inferior protocerebrum
PFA	Paraformaldehyde
PI	Pars intercerebralis
Pit-1	Pituitary-specific positive transcription factor 1
Pitx1	Paired-like homeodomain transcription factor 1
PL	Pars lateralis
PLc	Contralateral PL
PLi	Ipsilateral PL
PLp	Proximal PL
POMC	Proopiomelanocortin
PSD-95	Postsynaptic density-95
RIA	Radioimmunoassay

sGC	Soluble guanylate cyclase
SMB	Sodium meta-bisulfite
SNP	Sodium nitroprusside
TB	Total bound
uNOS	Universal NOS
vL	Ventral lobe

1 Summary

Juvenile hormone (JH) and nitric oxide (NO) both influence the reproductive behavior of female grasshoppers. Allatectomized females that cannot produce JH anymore, either loose or never establish receptivity, depending on the time point of allatectomy, and do not stridulate for the rest of their life (Loher and Huber, 1964, *Gomphocerus rufus*). Inhibition of nitric oxide synthase (NOS) in the central complex disinhibits sound production in restrained females and systemic application of a NOS inhibitor promotes reproductive related sound production of freely moving *Chorthippus biguttulus* females (Weinrich et al., 2008).

This thesis explored a possible connection between JH and NO signaling in the control of reproductive behavior by manipulating JH levels and analyzing the consequences on grasshopper females' sound production and mating behavior and by neuroanatomical studies exploring a functional relation between JH and NO on a cellular basis.

Observation of the natural reproductive behavior of *Chorthippus biguttulus* females in small laboratory populations established a reliable temporal scheme for the succession of reproductive states that served as a basis for subsequent studies with manipulated animals. Imaginal molt is followed by a period of 'primary rejection' that after approximately one week changes directly into 'active copulatory readiness', associated with female sound production that serves as a strong male-attracting communication signal. Before the first oviposition approximately two weeks after imaginal molt, females mated multiply and displayed only short periods of secondary rejection in between during which they were mute and refused males' mating attempts.

In a series of behavioral experiments, JH levels were altered by chemical allatectomy and topical application of synthetic JH III. These experiments, in combination with measurements of JH III titers at various reproductive states of *Ch. biguttulus* females, demonstrated that JH is necessary to initiate and sustain reproductive behavior, but higher JH titers suppress female sound production.

Inhibition of NO production by systemic application of the NOS inhibitor aminoguani-

dine increased female sound production while lowering JH hemolymph titers. The same effect of NO inhibition was observed in adult *Diploptera punctata* females, suggesting that NO signaling stimulates JH production. This was supported by the detection of increased JH levels in female grasshoppers treated with a NO donor. The promotive effect of NOS inhibition on female grasshopper sound production that was observed in earlier studies may therefore be indirectly mediated by suppression of JH synthesis in the corpora allata.

Immunocytochemical studies allay any doubts concerning the possibility of NO serving as an intercellular signal within the corpora allata. NOS expressing cell bodies were labeled by NADPHdiaphorase staining, anti NOS immunocytochemistry and NOS inhibitor-sensitive accumulation of citrulline, a byproduct of NO production. RFamide immunoreactive brain-to-corpora allata projections from pars intercerebralis and lateralis neurons were identified as cellular targets of NO release in the corpora allata. Their cGMP accumulating fibers were reconstructed and found to invade the entire corpora allata tissue.

Comparison of reports from the literature with the results from my PhD study suggest the following role of NO signaling in the control of corpora allata function: an RFamide as the transmitter of brain-to-CA projection neurons in *Ch. biguttulus*, inhibits JH release (It is known that the corpora allata stand under inhibitory control by the brain). At the same time, the RFamide stimulates NO production by the JH generating corpora allata cells. NO serves as a feedback signal, and in response reduces RFamide release from synaptic terminals within the corpora allata which leads to an increase in JH production.

Involvement of NO/cGMP signaling in the regulation of corpora allata function, NO production and RFamide controlled hormone release by corpora allata cells parallels the situation in the vertebrate adenohypophysis where NO is, amongst others, produced by gonadotropes (Ceccatelli et al., 1993) and RFamides control the release of gonadotropic hormones (Tsuitsui et al., 2000; Navarro et al., 2005). These results of my PhD study supplement numerous other similarities between insect corpora allata and vertebrate adenohypophysis documented in the literature, including gene and protein expression, general function, and development and support the hypothesis that the two organs may be of common evolutionary origin.

2 General introduction

2.1 Reproductive behavior of *Chorthippus biguttulus* and its neuronal control

2.1.1 Mating behavior of *Chorthippus biguttulus*

The mating behavior of grasshoppers has been subject to various scientific approaches for many decades, reaching from descriptions of their acoustic communication system to the investigation of neural processes controlling this behavior. Males and females produce sounds by rubbing a row of pegs at the inner side of the hind legs rhythmically against a protruding vein at the forewings (von Helversen and von Helversen, 1997). This behavior, called stridulation, serves several communication purposes (Faber, 1929, 1932): Males produce different sound patterns depending on the behavioral situation. They attract the attention of a female by 'calling songs' or they express rivalry to other males by producing the 'rivalry song'. In the presence of a female, males produce the 'courtship song' which resembles the spontaneous calling song but is of lower amplitude and more irregular (von Helversen and von Helversen, 1975). Shortly before and during mating, males generate mating sounds (Faber, 1929). In *Ch. biguttulus*, the male calling song consists of phrases, lasting 2-3 seconds. These phrases are composed of syllables (50 ms) divided by short gaps (10-15 ms) (Elsner, 1974). A shift between the movement patterns of the two hindlegs camouflages the gaps within the syllables, leading to higher attractiveness of male calling songs. *Ch. biguttulus* female stridulation is very similar to the male calling song but is characterized by a lower sound intensity, shorter phrases and variable hind leg coordination (von Helversen and von Helversen, 1997). A receptive female responds to conspecific male calling songs, and the potential mating partners start duetting with alternating phrases (von Helversen and von Helversen, 1997) and the generation and coordination of hind leg singing movements (Heinrich et al., 1997).

This sender-responder-system attracted scientific interest. The coevolution of the male's stridulation pattern and the female's recognition scheme (von Helversen and von Helversen, 1994) or the consequences of female choice (Klappert and Reinhold, 2007) are frequently studied topics in addition to the neural mechanisms underlying acoustic pattern recognition and song production. In particular, these concern the auditory networks in the metathoracic ganglion where first processing of the auditory information takes place (Stumpner and Ronacher, 1991), recognition of sound frequency in the brain (Pollack and Imaizumi, 1999, insects in general), and mechanisms that control the stridulatory answer to a conspecific song (Hedwig and Heinrich, 1997).

2.1.2 Neuronal basis of stridulatory behavior

Many aspects of grasshopper auditory perception, metathoracic processing of auditory information, and the subsequent flow of information that eventually elicits a response song are quite well understood.

The auditory nerve casts the sensory information from the tympanal receptor cells to the primary auditory neuropil in the metathoracic ganglion. Already at the level of receptor neurons, a first enhancement of relevant communication signals takes place (Rokem et al., 2006) while the information is further processed in the primary auditory neuropil to accentuate and extract information about frequency content, temporal patterns and direction of the sound source (Stumpner and Ronacher, 1991) and sent to the brain via ascending neurons that branch in the lower lateral protocerebral neuropil (Hedwig, 1986). The eventual identification of a conspecific song based on its frequency content and temporal pattern is accomplished by the brain (Bauer and von Helversen, 1987) involving neurons that are exactly tuned to the song's frequency and amplitude modulation (Römer and Seikowski, 1985).

The decision to answer a conspecific song by own stridulation is made in the brain. Constricted grasshoppers could be stimulated to stridulate by microinjection of neuroactive substances into the central body, a neuropil in the middle of the protocerebrum (Heinrich et al., 2001). The output information of the central body is, directly or indi-

rectly, relayed to command neurons whose activity is mandatory for the performance of stridulation (Hedwig and Heinrich, 1997). These neurons with somata located at the midline of the protocerebral lobe and dendritic branchings in the medial dorsal neuropil send descending axons through the medial part of the contralateral connective to the central pattern generator in the metathoracic ganglion. In this ganglion, the neuronal activity pattern, necessary to create the motor output of a defined song type, is generated (Hedwig, 1986). The command neurons excite the network of the central pattern generator and thereby elicit and maintain stridulation.

2.1.3 Behavioral aspects of female stridulation

Behavioral experiments with artificial stimuli revealed that females mainly use the high intensity at a syllable's onset and the pauses between the syllables as cues to recognize a conspecific male's calling song (Balakrishnan et al., 2001). But females do not coercively answer every male calling song they recognize. The physiological state together with the actual behavioral situation of the female determines if stridulatory behavior is performed. Certain physiological conditions prevent female sound production irrespective of any other natural external stimuli (Kriegbaum, 1988). Von Helversen (1972) found the egg deposition cycle playing an important role in the stridulatory behavior of females: they do not answer to male stridulation a few hours before and after laying an egg pod. Other factors that strongly impact female stridulatory behavior are age and mating status.

It has often been assumed that the behavioral patterns of female grasshoppers generally follow a scheme designed after observations on *Euthystira brachyptera* (Renner, 1952) and *Gomphocerus rufus* (Loher and Huber, 1964). In the first days after their imaginal molt, females are in the phase of 'primary rejection'. They are unreceptive, fend off male mating attempts, and do not stridulate. This phase is followed by a few days of 'passive readiness'. Though the females tolerate mounting by a male and also the copulation, they do not answer to male calling songs and do not actively approach males. If copulation is prevented during this phase, females get into the phase of 'active readiness' that is characterized by answering to male stridulation, approaching males and allowing the copulation

without prolonged courtship of the male. After mating, the females fall into 'secondary rejection' in which they are silent again and repel mounting by a male. This phase can persist for several oviposition cycles.

2.2 Influences of nitric oxide on female behavior

2.2.1 The nitric oxide/cGMP system in the brain of vertebrates and invertebrates

In both, vertebrates and invertebrates, nitric oxide (NO) is generated by the nitric oxide synthase (NOS) from L-arginine that is converted to L-citrulline under consumption of O_2. Vertebrates possess three genes for NOS (*NOS1-3*). By posttranscriptional editing, these genes result in several subtypes of the enzyme which can be separated in the constitutive isoforms, activated by Ca^{2+}/calmodulin, and the inducible isoforms that are calcium independent (Davies, 2000). Insects possess only one gene for NOS. Nevertheless, many different NOSs can be produced by splicing. It was long believed that all insect isoforms resemble the vertebrate nNOS (Elphick et al., 1993; Davies, 2000). This isoform is activated by Ca^{2+}/calmodulin binding to the regulatory site which induces a conformational change (Crane et al., 1999). But also inducible NOS was found in *Anopheles stephensi* (Luckhart et al., 1998).

The nNOS is a dimer consisting of an oxigenase and a reductase domain. The reductase domain contains binding sites for flavin and NADPH and transfers electrons from NADPH to a heme group in the oxigenase domain which also possesses the binding site for L-arginine (Stuehr, 1997). In vertebrates, the nNOS additionally contains a PDZ motif which binds to the cytoskeletal scaffolding protein PSD-95 (postsynaptic density-95) clustering NMDA receptors in a way that glutamate mediated Ca^{2+} influx is directly coupled to NO production (Sattler et al., 1999). The invertebrate isoform of NOS lacks this motif (Davies, 2000). Here, nicotinic and muscarininc acetylcholine receptors are suggested to be coupled to the NO/cGMP system (Zayas et al., 2002; Trimmer and Qazi, 1996).

Being a gas, NO can cross membranes and freely diffuse through the tissue over dis-

tances of up to 400 μm (Ledo et al., 2005). One of its main targets is the soluble guanylate cyclase (sGC) which is activated by NO and synthesizes cyclic GMP in response. Cyclic GMP as a second messenger can modulate ion channels and protein kinases and thereby change the excitability of neurons (Prast and Philippu, 2001). Another effect of NO is the inhibition of mitochondrial cytochrome c oxidase what leads to reduced cell respiration (Moncada and Bolaños, 2006).

Even though vertebrates and invertebrates are not closely related, there is conserved NO signaling in numerous brain functions, like olfaction (Kishimoto et al., 1993; Elphick et al., 1995), vision (Bredt et al., 1990; Elphick et al., 1996), and learning and memory (Hölscher and Rose, 1992; Jaffe and Blanco, 1994).

2.2.2 Nitric oxide suppresses sound production in females

Stridulation is controlled by the central body complex where the strength of inhibitory and excitatory signaling pathways decides about the performance of this behavior (Heinrich et al., 1997, 1998). In males, stridulation can be induced by a release from inhibition as has been done by injection of picrotoxin, an antagonist to GABA and glycine activated Cl^- channels, into the central body region (Heinrich et al., 1998). From this experiment, it can be concluded that some excitatory signaling that promotes stridulation is probably permanently present. In addition, stridulatory behavior is actively inhibited by suppressors when it is inappropriate. Regardless of some open aspects in female behavior, agreement prevails that females of many stridulating grasshopper species sing rarely spontaneously and less often than males. As stridulation is actively inhibited in males, a suppressing substance might prevent the performance of stridulation during the silent phases in females, as well.

A candidate to be this inhibitory substance is nitric oxide which is released by neurons in the central complex (Weinrich et al., 2008). By injection of the NOS inhibitor aminoguanidine into the central complex of females, stridulation that resembled the natural song in pattern and syllable duration could be induced (Weinrich et al., 2008). As von Helversen (1972) reported, virgin females show a reduction in their stridulation motivation

around the time of egg deposition. When intact virgin females were injected systemically with the NOS inhibitor aminoguanidine (AG) dissolved in locusta saline, NO production in their brain was reduced, indicated by stainings against citrulline, the byproduct of NO synthesis. Females treated with AG answered longer and more frequently to male calling songs and in some cases even shortly before and after laying an egg pod (Weinrich et al., 2008). This study shows that disinhibition by interference with NO release in the central complex promotes sound production in *Ch. biguttulus* females.

2.3 Regulation of female reproductive behavior by juvenile hormone

2.3.1 Juvenile hormone in insects

The first hints to a hormone secreted by the corpora allata inhibiting precocious metamorphosis in juvenile insects and regulating reproduction in adults came from Wigglesworth (1936). He reported about an inhibitory hormone that compensates the effects of molting hormone in larvae and is necessary for the production of ripe eggs in female *Rhodnius prolixus*. Due to its "juvenile keeping" effect on larvae, the hormone was named juvenile hormone (JH). The structure of JH was resolved in 1967 (Röller et al., 1967). Until today, four different juvenile hormones have been identified in insects all belonging to the group of sesquiterpenoid epoxide methyl esters with JH III being the most prominent form that is present in all insect orders (Hartfelder, 2000).

In the parenchymal cells of the corpora allata, JH is synthesized via the mevalonate pathway that starts from acetyl CoA and leads to the formation of farnesoic acid. Beginning from farnesoic acid, JH can be generated via two pathways. One leading over juvenile hormone II acid, the other over methyl farnesoate. Due to its lipophilic character, JH can cross cell membranes and is released from the corpora allata immediately after its generation. In the hemolymph, JH is transported by lipophorins or hexameric proteins to its sites of action (Hartfelder, 2000).

The effects of JH are pleiotropic and diverse. Despite its lipophilic character, JH

interacts with membrane bound proteins as well as with cytoplasmic proteins (Wheeler and Nijhout, 2003) and can regulate the activity of many different genes (Flatt et al., 2005). Many behavioral and phenotypic features of insects are affected by JH and the exact way of action is still poorly understood (Flatt et al., 2005). But recently, Zhu et al. (2010) demonstrated that JH increases gene expression in female mosquitoes and could also identify target genes.

2.3.2 Effects on female behavior

JH seems to be *the* master regulator for female reproductive behavior (Hartfelder, 2000). In social insects like *Bombus terrestris* and *Apis mellifera*, the JH titer and the rate of vitellogenin synthesis determine the caste status that defines an individual's reproductive activity (Larrere and Couillaud, 1993; Robinson et al., 1991). JH regulates the release of pheromones in cockroaches (Schal et al., 1997) and modulates the phonotactic behavior of cricket females (Stout et al., 1991). Female grasshoppers (*Gomphocerus rufus*) that lack JH after removal of the corpora allata do not stridulate and refuse mating with a male for their entire life (Loher, 1962).

The rate of JH synthesis is correlated to the behavioral status of adult female grasshoppers (Hartmann et al., 1994, *G. rufus*). Before mating, the in vitro synthesis of singing virgins reached the highest values, while the synthesis rate of silent virgins in the phase of 'passive readiness' was very low. Mating, the trigger signal to initiate 'secondary rejection', reduced JH synthesis rates to low levels.

But not the synthesis rate of JH alone is important, also the time point of the highest JH titer in the hemolymph determines a female's reproductive state. By applying certain diets, Hatle et al. (2000) could shift the timepoint of the highest JH titer in female grasshoppers and show that oviposition occurs always in the same temporal distance to the peak in JH titer. As a gonadotropin, JH stimulates the synthesis of vitellogenin by the fat body and its uptake by the oocytes (Chen et al., 1979). The JH titer thereby influences egg maturation (Schmidt and Othman, 1993) and can shift the timepoint of oviposition (Min, 2003).

2.3.3 Control of JH synthesis

Via the nervi corporis allati I and II, NCAI and NCAII, the corpora allata receive innervation from the brain (Vullings et al., 1999) and the subesophageal ganglion (Mason, 1973). Most of the direct connections from the brain have their origin in the neurosecretory pars intercerebralis and pars lateralis and run through the nervus corporis cardiaci II through the corpora cardiaca and the NCAI. Synthesis of JH is not only regulated by chemical signals from the brain, also ovarian factors in the hemolymph influence the activity of the CA as part of a feedback loop (Elliott et al., 2006).

For the largely unresolved control of JH synthesis by the brain both, humoral and synaptic pathways, are conceivable. Neurosecretory cells could secrete factors into the hemolymph that affect the CA or substances are synaptically conveyed to the parenchymal cells by axon terminals branching within the CA (de Kort and Granger, 1981).

Glutamate regulation of JH synthesis can be an example for synaptic transmission within the CA. The activation of insect NMDA receptors on CA cells by glutamate leads to an influx of Ca^{2+} that can stimulate JH synthesis (Chiang et al., 2002). On the other hand, glutamate also inhibits JH generation by the opening of glutamate gated chloride channels (Liu et al., 2005). Both are present in the CA of female *Diploptera punctata* but the effect of glutamate is dependent on female age and the sensitivity to allatostatin (Liu et al., 2005). So far it seems unclear which receptor predominantly influences JH synthesis (Liu et al., 2005).

Peptidergic brain factors that affect JH synthesis are generally classified as stimulatory allatotropins and inhibitory allatostatins (Gilbert et al., 2000). In case of the allatotropins, only one peptide from *Manduca sexta*, the Mas-AT, could be identified (Kataoka et al., 1989). But its stimulatory effect occured only in Lepidopteran species and in the honeybee *Apis mellifera*, not in the cockroach *Periplaneta americana* and in the grasshopper *Schistocerca nitens*. Also none of the Mas-AT related peptides isolated from other insect species exerted an inhibitory effect on JH production (Homberg et al., 2004). Other substances had a stimulatory effect in vivo but failed to increase JH generation by direct application to the CA. A possible explanation is an inhibitory effect on

allatostatin releasing neurons that leads to a release of inhibition and thereby increases JH synthesis, reviewed by Gilbert et al. (2000).

Compared to allatotropins, much more is known about the allatostatins (ASTs) (Stay and Tobe, 2007, review). They can be assigned to three different families, the A-ASTs, B-ASTs, and C-ASTs, whose members have certain amino acid sequences in common and could be detected in many different insect taxa. ASTs influence JH synthesis most probably in a paracrine mode, when they are released by terminals of nerves branching within the CA. Although receptors for ASTs were identified in *Drosophila*, it is not exactly known how JH synthesis is inhibited by ASTs.

Allatostatins and -tropins are not the only brain derived substances that regulate JH synthesis. Many neuropeptides and biogenic amines like FMRFamide (Carroll et al., 1986), CCAP (Dircksen and Homberg, 1995), proctolin (Clark et al., 2006), octopamine, and dopamine (Gilbert et al., 2000, review) were found in the CA, and some of them seem to interact with allatostatin containing neurons (Stay and Tobe, 2007).

JH titers in the hemolymph are also regulated by degradation by JH esterase and sequestration by JH binding proteins in the hemolymph (Nijhout and Reed, 2008) and are not always correlated to the activity of the CA (Bloch et al., 2000).

2.4 Aims of the thesis

2.4.1 Natural behavior of female *Ch. biguttulus*

The reproductive behavior of grasshopper females has been observed and described by various authors (Faber, 1929, 1932; Jacobs, 1950; Renner, 1952; Loher and Huber, 1964), which generated partially contradictory information by summarizing data from studies with different species.

In order to study the effects of experimental manipulations on the reproductive behavior of female *Ch. biguttulus*, it was necessary to elucidate their natural behavior in more detail. With respect to the occurrence of reproduction related physiological states it was in particular necessary to clarify the following aspects: When do *Ch. biguttulus*

females stridulate for the first time, do they express passive readiness, and how long does the phase of secondary rejection in this species last?

2.4.2 Regulation of female *Ch. biguttulus* behavior by juvenile hormone

As demonstrated by Hartmann et al. (1994) for the species *G. rufus*, the JH synthesizing activity of the corpora allata depends on a female's reproductive state. Since allatectomy leads to life long rejective behavior (Loher and Huber, 1964), JH seems to regulate reproductive behaviors that are associated with particular physiological states. Most likely *Ch. biguttulus* females also change their reproductive behavior upon interfering with JH signaling. This hypothesis was evaluated by measuring the JH titer in the hemolymph during different ages and in different reproductive states. The measurements of the JH titer in the hemolymph were complemented by behavioral experiments. In those experiments, females were chemically allatectomized by the application of precocene I. Their stridulatory and mating behavior were analyzed and compared to a control group. Subsequently, allatectomized females were treated with supplemental JH III to find out if exogenously applied JH could restore the effects evoked by allatectomy.

2.4.3 Relationship between JH control and the NO/cGMP system

JH and the NO/cGMP system in the brain both influence the same behavior in the female: stridulation to promote mating. NO is probably permanently released in the central body, the control center for stridulation, and inhibits this behavior in situations where sound production is unfavorable (Weinrich et al., 2008). JH has a similarly strong influence on the performance of stridulatory behavior (Loher and Huber, 1964; Hartmann et al., 1994). Since the ultimate decision to perform stridulation is made by the central body complex, a relationship between this structure and the JH control system is conceivable.

There are already speculations that NO could influence the juvenile hormone production. Kurylas et al. (2005, *Schistocerca gregaria*) assumed colocalizations of allatostatin or allatotropin with NOS and NADPHd in cerebral neurons and Skinner et al. (2000, *Diploptera punctata*) found allatostatin positive fibers in very close proximity to NOS

containing fibers in the CC. Chiang et al. (2000) correlated NADPHd activity in the corpora allata to mating in *Diploptera punctata* and found a change in activity only in mated females. NO could indirectly influence JH production by changing the activity of allatostatin or -tropin containing neurons or the expression of these peptides. Alternatively, JH could have effects that modulate NO/cGMP signaling in the brain, particularly in the central complex.

By double stainings against different allatoregulatory peptides and components of the NO/cGMP system in the brain, possible relations between the two systems were evaluated. With different backfill techniques, the origin of NO producing fibers in the CA was traced.

3 Behavioral experiments

3.1 Reproductive behavior of female *Ch. biguttulus*

The following chapter about the natural behavior of female grasshoppers was published in Journal of Insect Physiology (Wirmer et al., 2010). Printing in this thesis was approved by the editors. My own contribution to this paper included the development of the experiments, conducting most of the experiments with grasshoppers in small laboratory populations, part of the experiments concerning male stimulated stridulation of female grasshoppers, evaluation of video material, analysis of data from all experiments, and writing the raw version of the manuscript.

3.1.1 Introduction

In most gomphocerine grasshoppers both males and females produce acoustic communication signals by rhythmically rubbing their hind legs against the forewings (Elsner, 1974). Grasshopper acoustic signals are species- and sex-specific and serve to recognize, select, and localize reproductive partners. In some species including *Chorthippus biguttulus*, the mode of acoustic communication is determined by the reproductive state of the female which depends on maturation, sexual activity, and oviposition cycles (Renner, 1952; Kriegbaum and von Helversen, 1992; von Helversen, 1972). Based on experimental data derived from studies on a few species (see below), it has been assumed that the reproductive period of virgin grasshopper females can generally be subdivided in two parts, 'passive copulatory readiness' and 'active copulatory readiness'. In the state of 'passive copulatory readiness', females are mute and tolerate male mating attempts after courtship (Loher and Huber, 1964; von Helversen and von Helversen, 1983; Hartmann and Loher, 1996). This unilateral communication switches to bilateral communication when females enter 'active copulatory readiness' (Loher and Huber, 1964). In this state, the female sings spontaneously or answers to male calling songs which may initiate prolonged alternating duets with the male during which phonotaxis is performed by the male (von Helversen and von Helversen, 1983).

The course of female grasshopper reproductive states has been studied in a rather small number of species including *Gomphocerus rufus* (Loher, 1962; Loher and Huber, 1964; Riede, 1983), *Euthystira brachyptera* (Renner, 1952) and some specific aspects more recently in *Chorthippus parallelus* (Kriegbaum, 1988; Reinhardt and Köhler, 1999; Reinhardt, 2007). Although some differences between these species have been reported, the following scheme for the general course of female reproductive states has been widely accepted and used as a basis for studies with other grasshopper species (Fig.1). During the first days after their imaginal molt, females do not stridulate and fend off male mating attempts. This behavioral state was named 'primary defense' by Loher and Huber (1964) but we suggest to denote it 'primary rejection' in order to discriminate it from the term used to describe predator-prey behavior (Edmunds, 1974). After a few days, females assume the state of passive readiness during which they remain silent but may mate after male courtship. If females do not copulate during passive readiness, they enter the state of 'active copulatory readiness' in which they respond to male calling songs or even stridulate spontaneously and accept male mating attempts without prolonged periods of courtship. After mating, females either assume a state of 'secondary rejection' during which they reject male mating attempts (Hartmann and Loher, 1974, 1996, *G. rufus*) or, in species where subsequent copulations are prevented by the spermatophore plugging the spermathecal duct, display a period of 'passive copulatory readiness' that changes to 'secondary rejection' shortly before the first oviposition (Renner, 1952, *E. brachyptera*), (Hartmann and Loher, 1974, *Chorthippus curtipennis*). Studies on other species reported that females copulated multiple times in rather short intervals (Kriegbaum, 1988; Reinhardt and Köhler, 1999) and responsiveness of virgin females to male songs either increased (Loher and Huber, 1964; Kriegbaum and von Helversen, 1992) or decreased (Reinhold et al., 2002) with age. These observations suggested that the general scheme for the course of female reproductive states and associated behaviors cannot be extrapolated to all gomphocerine grasshoppers.

The nightingale grasshopper *Ch. biguttulus* (L.) is a frequently used species for neurobiological, behavioral, ecological, evolutionary, and hormone physiological studies.

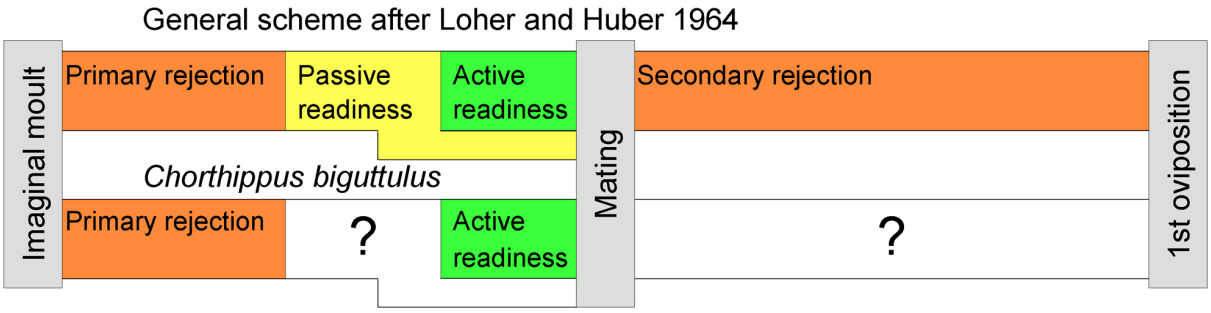

Figure 1: *Reproductive states of acoustically communicating grasshopper females between imaginal molt and first oviposition*
Upper part summarizes a scheme that was proposed by Loher and Huber (1964) based on experimental data from studies on *Gomphocerus rufus* and *Euthystira brachyptera*. Detailed explanation is provided in the text. Various studies on other grasshoppers including *Chorthippus biguttulus* indicated that this general scheme may not apply to all acoustically communicating species, questioning the occurrence of 'passive copulatory readiness' and the nature of behavioral states that follow mating in particular.

Male calling and courtship songs and female response songs that serve as crucial elements in the reproductive behavior of *Ch. biguttulus* (von Helversen and von Helversen, 1997) have been employed for studies intended to understand bioacoustic phenomena in the field (Lang, 2000; Gilbert and Elsner, 2000), criteria for female choice (Kriegbaum, 1989; Kriegbaum and von Helversen, 1992; Reinhold et al., 2002; Safi et al., 2006), localization and recognition of acoustic patterns (von Helversen and von Helversen, 1997; Balakrishnan et al., 2001; Schmidt et al., 2008), mechanisms of auditory information coding in the nervous system (Machens et al., 2001, 2003; Neuhofer et al., 2008; Ronacher et al., 2008), and the neuropharmacological basis of motivational states (Heinrich et al., 2001; Hoffmann et al., 2007; Weinrich et al., 2008). In contrast to numerous studies on the production and processing of reproduction-related acoustic signals of *Ch. biguttulus*, general information about its reproductive states and associated behaviors are merely derived from observations on other species (e.g. *G. rufus*, *E. brachyptera*, *Ch. parallelus*, *Ch. curtipennis*), although, these studies revealed considerable differences (mentioned above) in the course of female reproductive states. In order to provide information about the reproductive behavior of this important grasshopper species, we studied the course of reproductive behavioral states of individually labeled *Ch. biguttulus* females in small laboratory populations between their imaginal molt and first oviposition. In particular, we asked the questions, whether *Ch. biguttulus* females express a period of 'passive readiness',

whether they re-establish copulatory readiness after matings and whether they copulate more than once prior to first oviposition. The results are compared with data from earlier laboratory and field studies on *Ch. biguttulus* and other grasshopper species.

3.1.2 Materials and methods

Animals

Studies were performed with adult male and female grasshoppers of the species *Ch. biguttulus* (Orthoptera: Acrididae; L. 1758). All females used in this study were reared from eggs that derived from grasshoppers collected in the vicinity of Göttingen, Germany in the previous summer (the summers of 2007 and 2008). Mixed populations of these grasshoppers were kept for up to 3 weeks in the laboratory enabling females to lay eggs into dishes filled with moistened vermiculite. Male grasshoppers used in our study either derived from the same clutches or were caught as subadults in grassland areas around Göttingen. Eggs were kept at 4°C for at least 4 months. Hatching occurred after approximately 1 week at 26°C and the nymphs were fed with grass and supplemental food for crickets (Nekton, Pforzheim, Germany) ad libitum. After molting into the fourth nymphal instar, fully intact females were randomly selected from the rearing cages and separated from males. The grasshoppers were reared and maintained at 20-26°C and photoperiods of 16 h light and 8 h darkness.

Determination of males' mating readiness

In order to select *Ch. biguttulus* males with high mating readiness as potential reproductive partners in subsequent experiments, we stimulated 14 mature males on 6 consecutive days after their isolation from females with the recording of a female song. Individual male grasshoppers were transferred to an arena that was heated by a heating lamp from above to constant 30°C. The record of female stridulation was presented at an intensity of 60 dB SPL to the right or to the left side of the body via a loudspeaker (Sound Craft Conrad DT 25 P). The acoustic stimulus consisted of 27 repetitions of a female song sequence of 1.5 s duration separated by 3.3 s long pauses. Recognition of female songs

elicits a uniform series of male reactions (von Helversen and von Helversen, 1983). We rated the intensities of the males' reactions to stimulation with female song with the following scores: 0, no reaction; 1, stridulation; 2, turning toward the sound source; 3, jumping or walking toward the sound source. The maximal response of each individual male on each day following separation from females was determined, and the days were ranked with respect to the maximal response intensities (higher ranks accord to higher maximal responses; equal ranks were assigned to days with equal maximal responses). After an overall Friedman test, a post hoc Wilcoxon-Wilcox test for multiple comparisons of matched samples was applied to compare the rank sums of the different experimental days. Males used in this experiment were not included in the experiments with females described above.

Reproductive behavior of Ch. biguttulus females in small laboratory populations

The behavior of six mature males and five individually marked females (starting with the first or second day after their imaginal molt) was recorded with a digital video camcorder (MV10, Canon) for 62 min between '1300 hours' and '1500 hours' on each day of the experimental period. This series of observations was repeated five times under equal conditions with different groups of females to obtain data for $N = 25$ females. Between daily observation periods, 15 females (three groups) were kept as a group with the opportunity to lay eggs into containers filled with vermiculite. Ten female (two groups) were kept in individual cages to determine their exact days of oviposition under rearing conditions in our laboratory. Earliest ovipositions in all five groups occurred at an age of 14 days but four of the individually housed females did not lay eggs until day 17, the longest period of observation. All females used in our experiments performed their first ovipositions later than day 13 (= last day of behavioral observation) after imaginal molt. Analysis of videotaped behaviors included (1) numbers of male mating attempts, (2) number and duration of mountings of females by males and (3) stridulatory activity of the females. Male mating attempts were counted as such if the male tried to mount the female after producing one or several sequences of courtship song in the

vicinity of the female (Faber, 1929). One female died during the experiment and was replaced by a younger one. Outside daily observation times, males and females were kept in separate rooms. Grasshoppers were individually marked with acrylic colours (Lukas CRYL TERTIA). Experiments took place in a cage with a base area of 12.5 cm × 9.5 cm and a height of 5.5 cm. The cage was heated to constant 30°C with a heat lamp and additionally illuminated with two light bulbs. For analysis, videos were later reviewed on a monitor (HR Trinitron, SONY) allowing the identification of previously described components of reproductive behavior. We evaluated the occurrence of male courtships, female stridulation, female rejections or failures of male mounting attempts, and successful mountings of females by males. Mountings were counted as successful matings if they lasted longer than 2 min. The five groups of females were tested on 13 consecutive days resulting in a total of 65 observation periods. On a single experimental day, the different groups of females encountered different males that had been isolated from females for at least 3 days. All experimental data were analyzed with Excel (Microsoft) and SPSS PASW Statistics 18.0 software (SPSS Inc., Chicago, US).

Evaluation of female behavior in small laboratory populations

To evaluate potential differences between the first occurrence of stridulation and the first occurrence of mounting or mating, respectively, those females that stridulated and allowed mounting and mating were analyzed in a generalized estimating equations (GEE) model. The GEE allows repeated observations of the same individuals nested in a hierarchy and the entry of not normally distributed data. The data were fitted to a Poisson distribution with a log-link function. Over- or underdispersion was eliminated by choosing a robust estimation of covariance. In the GEE, female behavior (first mounting, first mating, first stridulation) was included as a within subject factor with a first-order autoregressive working correlation matrix. Female identity nested within an experimental group (one of the five populations) was entered as a subject effect.

Male song – stimulated stridulation of virgin and mated grasshopper females

The responsiveness of *Ch. biguttulus* females to male calling song has previously been used to assess female reproductive readiness (von Helversen, 1972; von Helversen and von Helversen, 1997). To study the potential re-establishment of female 'active copulatory readiness' after copulation, *Ch. biguttulus* females were placed into a 15 cm × 12 cm × 12 cm observation cage heated to 30°C and stimulated with recordings of a male calling song. The acoustic stimulus that included five repetitions (separated by 5 s pauses) of a calling song containing three sequences (entire duration: 10 s) had previously been used to assess female responsiveness (Weinrich et al., 2008). It was presented at a volume of 65 dB SPL via a loudspeaker (Sound Craft Conrad DT 25 P) that was positioned at a distance of 10 cm beside the cage. Initially, 24 virgin females that responded to the male calling song were individually placed together with three intact, highly motivated males to mate with one of them. Matings were assessed as successful, if a connection between the sexual organs was maintained for a minimum of 2 min and the female did not answer to male calling songs 5 min after the end of the copulation. The responsiveness of 24 females to male calling song was tested on 5 consecutive days following the day on which copulation occurred. For eight of these females, male song-stimulated stridulation was recorded with a digital camera (Digital Video Camcorder MV 10; Canon) in order to quantify the numbers and duration of female song sequences. Spermathecae of all females were checked for sperm content 6 days after mating at the end of the experiments to confirm successful mating (see below).

Detection of sperm in spermathecae

Grasshopper females were anesthetized by cooling. The spermatheca was removed and fixed overnight in 0.1 M PO_4 buffer (pH 7.4), containing 4% paraformaldehyde at 4°C. After fixation, the spermatheca was embedded in gelatine albumine followed by another overnight fixation in 4% paraformaldehyde in 0.1 M PO_4 buffer (pH 7.4). Gelatine albumine blocks were briefly rinsed with PO_4 buffer and cut with a vibratome (LEICA, VT1000S) into 50 μm thick sections. Those were rinsed with PO_4 buffer

overnight. After blocking with 1% normal goat serum (Amersham Biosciences), 0.5% bovine serum albumine (MP Biomedicals Inc.), and 1% Triton X-100 (Sigma-Aldrich) in 0.1 M PO_4 buffer for 3 h, the first antibody directed against acetylated α-tubulin (Sigma, T-6793, monoclonal mouse) was applied 1:1000 in blocking buffer for 17 h at 4°C. After washing in PO_4 buffer, the secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (Invitrogen), was applied 1:100 in blocking buffer for 3 h. Sections were rinsed with PO_4 buffer several times, transferred to a mixture of PO_4 with glycerol (1:1), and mounted on microscope slides. Immunofluorescence was analyzed with a confocal laser scan microscope (LEICA TCS SP2). Maximal intensities of image stacks were extracted with Leica Confocal Software Lite. Brightness and contrast of projections were adjusted with Photoshop (Adobe). Sperm was identified after the description of (Winterton et al., 1999).

Impact of female stridulation on male mating readiness

Every female was tested on 13 consecutive days after imaginal molt. For each day, the mating attempts directed toward a specific female were counted. For each observation period the total number of stridulating females in the experimental cage was determined and it was noted, whether the particular female itself was stridulating. Data were analyzed in a generalized estimating equations (GEE) model. Due to overdispersion the data were fitted to a negative binomial distribution with a log-link function and a robust estimation of covariance was chosen. 'Behavior' (female is stridulating: yes = 1/no = 0) was defined as a factor in the model, age (day after the female's imaginal molt) and singers (number of stridulating females in the cage) as covariates. In addition, it was tested for interaction effects between the total number of singers in the cage and 'behavior' and between the age of the females and 'behavior'. Female identity nested within an experimental group was entered as a subject effect. For the within subject factor 'age', a first-order autoregressive working correlation matrix was chosen. The effect of factors/covariates on the number of male mating attempts was assessed.

3.1.3 Results

Mating readiness of male Ch. biguttulus

We analyzed the behavioral responses of males to stimulation with female songs on 6 consecutive days after their isolation from females. Over the first 3 days after separation from females, the number of unresponsive males declined (white bars) while the number of males displaying intensive responses (turning towards (dark gray bars) and approaching the sound source (black bars)) increased (Fig. 2a). Analysis of response intensities of all 14 males tested with a Friedman test revealed significant differences ($\chi^2_6 = 23.55$, $N = 14$, $P \leq 0.05$) of males' repsonsiveness after different times of isolation from females and a post hoc Wilcoxon-Wilcox test for multiple comparisons of matched samples identified male performance on day 1 to be significantly different from day 3 ($T = 29$, $N = 14$, $P \leq 0.05$) and day 6 ($T = 32.5$, $N = 14$, $P \leq 0.05$) (Fig. 2b). The results suggest that the males' reproductive readiness increases during the first 3 days of isolation from females and subsequently remains on this elevated level. In order to assure a high and comparable mating readiness, males were isolated from females for at least 3 days before they were used in the following experiments.

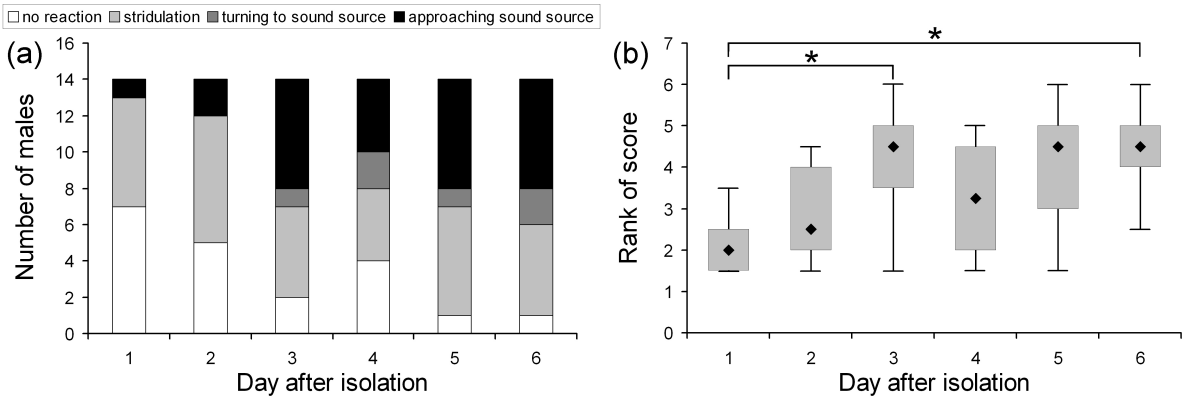

Figure 2: *Effect of isolation from females on male* Chorthippus biguttulus *reproductive readiness*
(a) The proportion of males that specifically reacted to the female song increased with increasing duration of isolation. From the third day on, the proportions of males that stridulated only and those that in addition turned to the sound source and approached it, remained approximately the same. (b) Average scores of responses of 14 males. Responses were scored as follows: no reaction = 0; stridulation only = 1; stridulation and turning to the sound source = 2; stridulation, turning and approaching the sound source = 3. (diamond) Median of ranks; boxes include upper and lower quartile of values; whiskers include all values; (∗) $P = 0.05$.

Reproductive behavior of Ch. biguttulus females in small laboratory populations

First stridulation of females was observed at adult age of 3 days. By day 13, 1 day before first ovipositions were recognized, 92% of the females had stridulated at least once. Until day 13 after imaginal molt, 88% of the females permitted mounting by a male. Since previous studies suggested that short mountings of only a few seconds do not lead to successful copulation, we separately evaluated the first occurrences of mountings that lasted for more than 2 min. These presumably complete matings were permitted by 68% of the females.

17 of the 25 females tested in five separate experimental groups started stridulation and allowed both mounting by a male and complete mating. The factor 'behavior' had a significant effect on the dependent variable 'age' (GEE: $\chi^2_2 = 8.188$, $P = 0.017$). Females stridulated for the first time with $X + SE = 5.94 + 0.43$ days and allowed short mountings by males with $X + SE = 6.00 + 0.51$ days. A significant difference between these time points was not detected (Wald $\chi^2_1 = 0.025$, $P = 0.875$, 95% $CI = 0.893$, 1.142 days). First matings, lasting more than 2 min, occurred with $X + SE = 7.65 + 0.65$ days and thereby significantly later than first stridulation (Wald $\chi^2_1 = 20.356$, $P \leq 0.001$).

Until the age of 13 days after molting and before the first oviposition, 10 of the 17 females (59%) that copulated for more than 2 min remated up to six times (Fig. 3). The average interval between two matings was 1.5 days ($X + SE = 1.5 + 0.46$ days, $N =$ 20 intervals evaluated). In two cases, females mated again on the next day following the previous mating and in eight cases females copulated twice or even three times during the observation period of a particular day. Due to the coincidence of first matings in some females and rematings in others, mating activity in the small laboratory populations reached its highest level at female ages of 7 days after imaginal molt. The durations of females' first and subsequent matings were compared with a paired t-test after the D'Agostino Pearson omnibus test detected no strong deviations from normal distribution ($K^2_{1st} = 1.98$, $K^2_{2nd} = 4.38$). Although, first matings lasted only $X + SE = 12.6 + 2.77$ min and secondary matings $X + SE = 20.9 + 6.61$ min, a significant difference was not found ($t_9 =$ -1.28, $N = 10$, $P = 0.232$; confidence interval: $d =$ -8.65 min, 95% CI: -23.92,

Day after imaginal moult	1	2	3	4	5	6	7	8	9	10	11	12	13	∑
Animal														
1				1					1					2
2														0
3				1			2						1	4
4											2			2
5						1	3	2						6
6							1							1
7									1					1
8							1			1				2
9							1							1
10														0
11						1						2		3
12								1						1
13														0
14							1	1			3			5
15														0
16							1							1
17												1	1	2
18														0
19														0
20													2	2
21								1						1
22							1							1
23														0
24														0
25												2		2
∑	0	0	0	2	0	2	11	5	2	1	5	5	4	37

Figure 3: *Observed matings in five small laboratory populations of six male and five female* Chorthippus biguttulus
Within 13 days after their imaginal molt, 17 of 25 individually labeled females copulated at least once and 10 of them twice or more (up to six) times without interspersed oviposition. Females started to copulate at an age of 4 days. In eight of the daily observation periods of 62 min, individual females copulated twice or even three times.

6.62 min).

Impact of female stridulation on male reproductive readiness

In order to evaluate whether female stridulation enhances male reproductive readiness, we analyzed the numbers of male mating attempts directed toward singing and silent females during daily observation periods in which different numbers of females in the experimental group were stridulating. The data were analyzed with a GEE model fitted for negative binomial distribution.

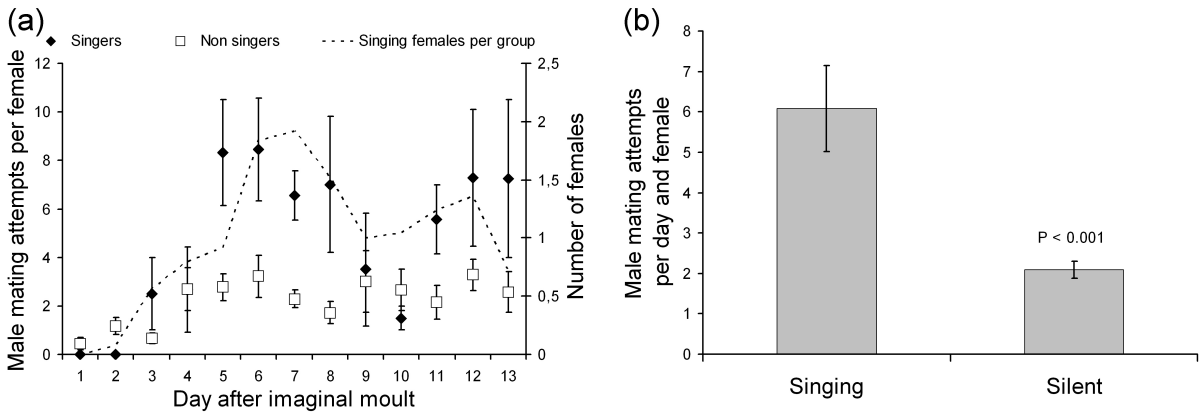

Figure 4: *Male mating attempts toward singing and mute females in small laboratory populations*
(a) Average numbers of mating attempts directed toward singing and mute females in relation to singing females per group at a particular female age (background stridulation). Error bars: SEM. Analysis by GEE revealed that actively stridulating females attracted more mating attempts than silent females and also the total number of stridulating females in the observation cage had a significant influence on the average frequency of male mating attempts. At a female age where relatively high background stridulation occurred, the difference in mating attempts between singing and silent females became even more pronounced. (b) Average numbers of mating attempts received by individual females. Females received significantly more mating attempts during observation periods in which they stridulated than during periods when they remained mute. Error bars: SEM.

The stridulatory behavior of individual females had a significant influence on the number of mating attempts performed by males in the model (GEE: $\chi^2_1 = 5.480$, $P = 0.019$). Stridulating females received $X + SE = 4.00 + 1.06$ mating attempts more than silent females (mating attempts toward singers: $X + SE = 6.08 + 1.06$; mating attempts toward non singers: $X + SE = 2.08 + 0.21$) (Fig. 4b). The cumulative stridulatory activity of all females in the cage also had a significant effect on male mating attempts (GEE: $\chi^2_1 = 6.220$, $P = 0.013$). With every additional singing female in the cage, males performed $X + SE = 1.63 + 1.09$ more mating attempts. An interaction effect between the females' behavior and the total number of singers in the cage was also present (GEE: $\chi^2_1 = 5.749$, $P = 0.017$). With each additional singing female in the cage, singing females received an average of $X + SE = 0.62 + 1.14$ additional mating attempts compared with silent females. Age of the female and repeated introduction of females and males to the small mixed populations had no detectabel influence on the number of male mating attempts. The results are illustrated in Fig. 4a.

Re − establishment of 'active copulatory readiness' in mated grasshopper females

Spermathecae of mated females contained anti-tubulin immunoreactive structures that were dispersed throughout the entire lumen and were interpreted as components of sperm (Fig. 5b). In contrast, lumina of fertilization organs of virgin females were entirely free of anti-tubulin immunoreactivity (Fig. 5a). We detected anti-tubulin immunoreactivity in

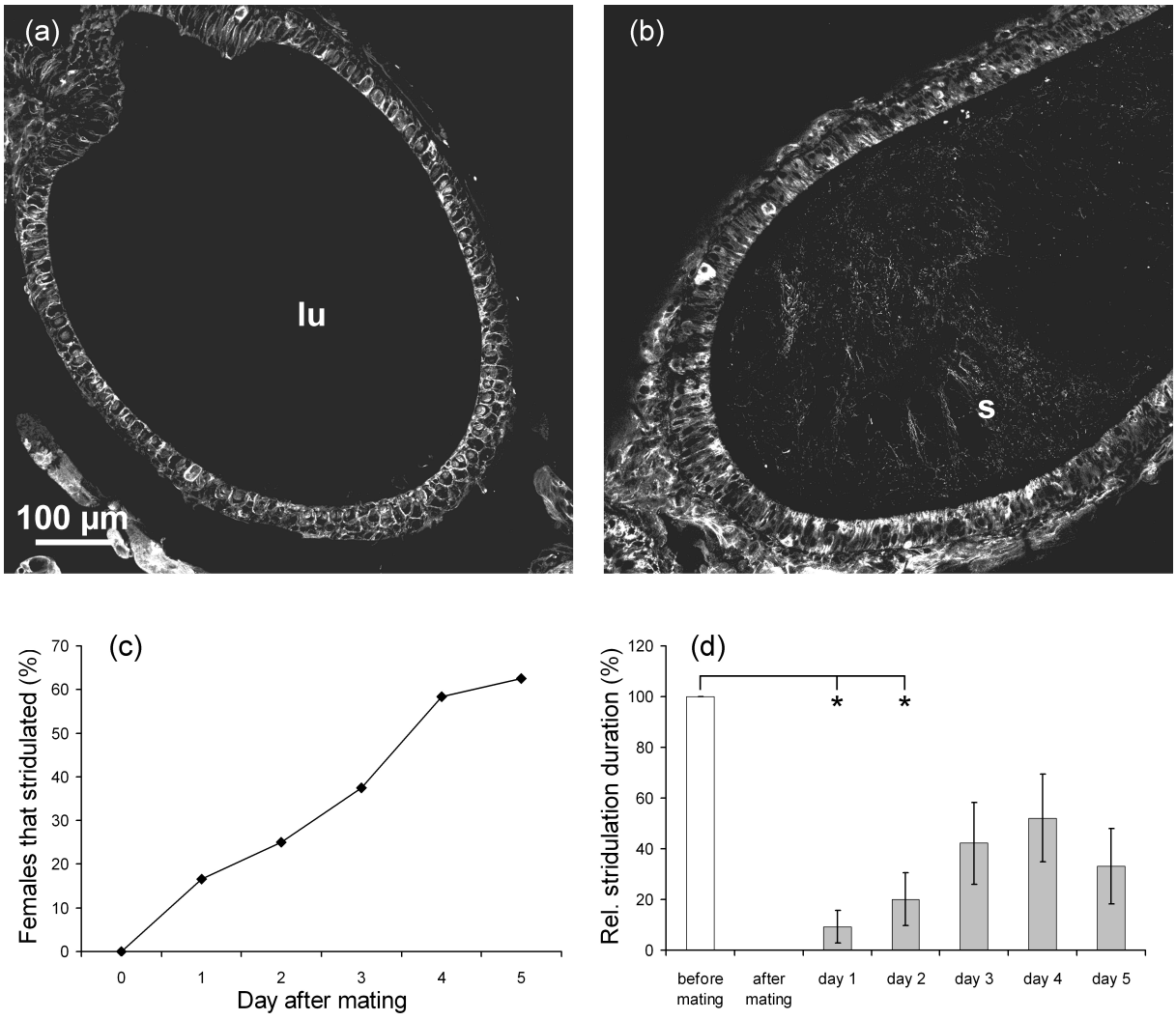

Figure 5: *Re-establishment of 'active copulatory readiness' in* Chorthippus biguttulus *females after copulation*
(a and b) Completed copulations were verified by the analysis of female bursae for sperm content on the sixth day following copulation. Photographs of histological sections through the bursa copulatrix show its lumen (lu) with surrounding epithel cells. While lumina of bursae copulatrix of virgin control females were unlabeled (a), all experimental females contained strong anti-tubulin immunoreactivity (s) that was indicative of sperm (b). (c) Proportion of females ($N = 24$) that performed male song-stimulated stridulation immediately after mating and on 5 subsequent days. (d) Relative duration of male song-stimulated sound production in females ($N = 8$) before mating, immediately after mating and on 5 subsequent days. The females' responsiveness was significantly reduced until the second day after mating (Friedman test, Wilcoxon-Wilcox test for multiple comparisons of matched samples).

the lumina of spermatheae of all 24 females that were used in this experiment, indicating that all received a spermatophore including sperm during the staged copulation at the beginning of the experiment.

Within 5 days after mating, 15 of 24 females (62.5%) responded at least once to acoustic stimulation with male calling song (Fig. 5c). Whether at all and after which period of time females re-established responsiveness was not correlated with the duration of copulation (data not shown). Eight of the 15 females that reacted with stridulation were part of a pre-selected group of females whose behavioral responses to acoustic stimulation before and after mating were videotaped for quantitative analysis (Fig. 5d). Females that did not respond to the male calling song 5 min after mating were tested for re-establishment of responsiveness on 5 subsequent days. On the first and second day after mating, two respectively three females responded to the male calling song and the overall duration of response songs was significantly reduced compared to the stridulatory activity before mating (Friedman test: $\chi^2_6 = 31.57$, $N = 8$, $P \leq 0.05$; Wilcoxon-Wilcox test for multiple comparisons of matched samples: day 1: $T = 34$, $N = 8$, $P \leq 0.05$; day 2: $T = 28$, $N = 8$, $P \leq 0.05$). On days 3-5 after mating, four or five females answered to male calling songs. Although the overall duration of response songs appeared to be lower than before mating, this difference was not statistically significant.

3.1.4 Discussion

Previous observation raised doubts whether the reproductive behavior of grasshopper females is similar among acoustically communicating species and follows a common pattern that has initially been formulated by Loher and Huber (1964) on the basis of their studies on *G. rufus* and studies of Renner (1952) on *E. brachyptera*. The present study on *Ch. biguttulus* females provides substantial evidence that their reproductive behavior differs considerably from this pattern (Fig. 6) which should be accounted for the interpretation of behavioral, neurophysiological and speciation studies performed with this species. Most importantly, *Ch. biguttulus* females do not assume a state of 'passive copulatory readiness', re-establish 'active copulatory readiness' after matings and may copulate multiple

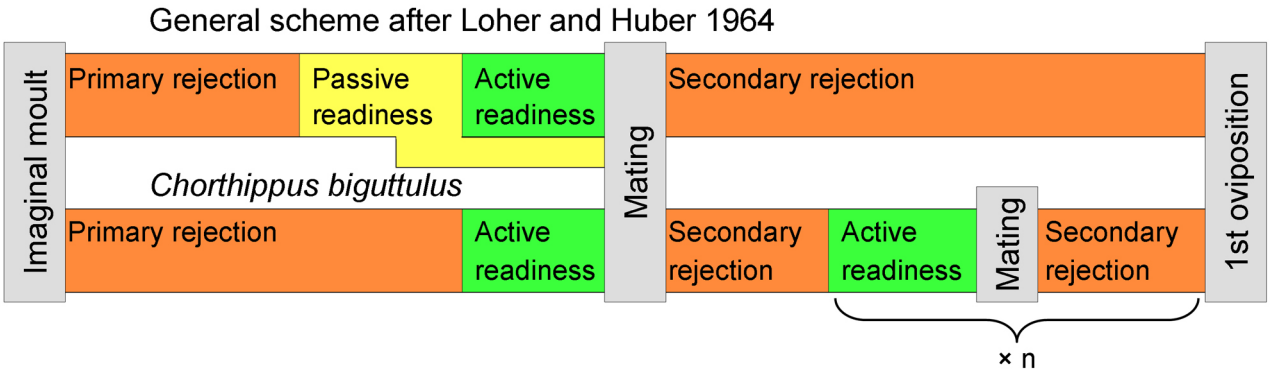

Figure 6: *Reproductive states of acoustically communicating grasshopper females between imaginal molt and first oviposition*
Upper part summarises a scheme that was proposed by Loher and Huber (1964) based on experimental data from studies on *Gomphocerus rufus* and *Euthystira brachyptera*. Details are explained in the text body to Fig. 1. In contrast to this scheme, *Ch. biguttulus* females do not assume a state of 'passive copulatory readiness', re-establish 'active copulatory readiness' after matings and may copulate multiple times before their first oviposition.

times before their first oviposition.

The state of 'passive copulatory readiness' is characterized by females being mute but permitting copulations with males after courtship. Observations of reproduction-related behavior of *Ch. biguttulus* females during daily periods of co-habitation with males revealed no difference between the ages when females stridulated for the first time and permitted male mountings (average age: 5.9 days vs. 6.0 days). In contrast, females were significantly younger when they started stridulating compared with the age when they performed first completed matings, which was indicated by copulation times longer than 2 min (average age: 5.9 days vs. 7.6 days). For comparison, a previous study by Kriegbaum and von Helversen (1992) with the same species reported first male song-stimulated stridulation at an average female age of 5.7 days. Though we always placed five females together with six males, whose reproductive readiness was high after at least 3 days of separation from females, not a single female mated before having stridulated at least once. This is even more remarkable, since studies on related species suggested that females in restricted spaces of laboratory populations may mate more frequently than females in field populations, because they cannot escape courting males (Reinhardt and Köhler, 1999; Arnqvist and Nilsson, 2000; Reinhardt, 2007). Since stridulation is a characteristic of 'active copulatory readiness', *Ch. biguttulus* females do not show 'passive

readiness' at all and the state of 'primary rejection' after their imaginal molt is directly followed by 'active readiness'. However, enhanced reproductive motivation in the state of 'active readiness' was not invariantly coupled to unselective acceptance of any courting male since stridulating females were observed to reject male mating attempts on several occasions. Similar observations were made during previous studies with *Ch. biguttulus* (Kriegbaum and von Helversen, 1992) and other species (Haskell, 1958; Loher and Huber, 1964). Similar to *Ch. biguttulus*, the species *Ch. parallelus*, *Chorthippus brunneus*, *Omocestus viridulus* and *Stenobothrus lineatus* were also mentioned to lack 'passive copulatory readiness' (Haskell, 1958; Loher and Huber, 1964) though we found no experimental data for this statement in the literature. In contrast, species like *G. rufus* and *E. brachyptera* displayed obligatory periods of 'passive copulatory readiness' that lasted for at least 1 day and were either terminated by copulation with a male or spontaneously changed into 'active copulatory readiness' (Renner, 1952; Loher, 1966).

The number of male mating attempts increased with the number of stridulating females in the experimental cage. Apparently, female stridulation serves as a stimulatory signal to males. Given the restricted space of the experimental cage, it was somewhat surprising that males were able to select the stridulating females for a directed phonotactic approach. Consequently, males distinguished stridulating from silent females and preferred singing females for mating attempts. This emphasises a function of female stridulation as a relevant communication signal, to attract males for copulation.

Previous studies disagreed on whether grasshopper females copulate multiple times before subsequent oviposition and how their behavior changes with and after mating (Renner, 1952; Hartmann and Loher, 1974, 1996; Butlin et al., 1987; Bella et al., 1992). Some authors reported that females assume a state of 'passive readiness' after mating and allow males to mount them but successful copulation is prevented by the spermatophore from the previous copulation. This spermatophore occludes the female's genital tract for 3-4 days post mating until close to the next oviposition (Renner, 1952, *E. brachyptera*) (Hartmann and Loher, 1974, *Ch. curtipennis*). In contrast, females of *G. rufus* eject the remainders of the spermatophore within a few hours after mating. Instead, a proteinous

component from the male accessory glands that is transmitted with the spermatophore during copulation induces the state of 'secondary rejection' in the female which lasts for 3-4 days and is usually terminated with oviposition (Hartmann and Loher, 1996). Furthermore, studies on laboratory populations of *Ch. parallelus* (Bella et al., 1992) and *Ch. brunneus* (Butlin et al., 1987) suggested that females of these species mate multiply without interspersed oviposition. The results from our experiments with *Ch. biguttulus* are in line with the latter studies and further indicate that females not only permit rematings but re-establish 'active copulatory readiness' after a short period of 'secondary rejection' following previous matings. By immunocytochemical detection of sperm in female bursae copulatrix, we confirmed the successful transmission of a spermatophore during first matings and excluded the possibility that only incomplete copulations lead to a re-establishment of 'active readiness'. Within 5 days after completed first matings, 15 out of 24 females (62.5%) responded at least once to the recording of male calling songs presented via a loudspeaker. Quantitative analysis of eight randomly selected females demonstrated, that females do not answer male calling songs immediately after mating, display significantly reduced responsiveness for 2 days following the day of mating, and maintain a reduced, though not significantly different, responsiveness on subsequent days, in comparison with their responsiveness before mating. Whether female responsiveness recovers to pre-mating levels or persists on a lower level due to reduced responsiveness around the time of oviposition (Skovmand and Pedersen, 1983; Hartmann and Loher, 1996, own observations from other studies) or any other reason, remains to be shown in an experimental series with a larger number of females. The potential presence of a sperm plug that mechanically interfered with subsequent successful copulations was excluded in *Ch. biguttulus*, since second copulations lasted as long as first copulations. Occlusion of the female genital tract in other species by remainders of the spermatophore leads to considerably shorter and unsuccessful second copulation attempts (Renner, 1952).

Ten out of 17 *Ch. biguttulus* females in small laboratory populations mated at least twice before their first oviposition and one female performed five rematings. Since all females demonstrated 'active copulatory readiness' by stridulation before rematings oc-

curred and females successfully rejected male mating attempts by hind leg kicking, it is unlikely that rematings were forced by the continuous presence of males in the observation cage. Therefore, and similar to *Ch. parallelus* (Bella et al., 1992; Reinhardt and Köhler, 1999) and *Ch. brunneus* (Butlin et al., 1987), *Ch. biguttulus* seems to be a multiply mating species. Multiple mating may repeatedly deliver nutrients to the female, leads to genetic variety of the offspring that may be beneficial under particular environmental conditions and is suggested to prevent replenishment of sperm for egg fertilization (Thornhill and Alcock, 1983). Studies on *Ch. parallelus* reported that one copulation transferred sufficient sperm for five to eight egg pods and some experimental females that copulated only once ran out of sperm for further fertilizations (Reinhardt and Köhler, 1999). While this study found no beneficial effects of multiple mating on offspring quality, another study on the same species (Reinhardt and Köhler, 1999) reported increased offspring weight, higher numbers of eggs per pod, and higher hatching and fertilization success after multiple compared to single copulations. With respect to potential mechanisms of sperm competition, studies on *Ch. biguttulus* and *Ch. parallelus* suggest that sperm from sequential copulations is either stratified within the spermatheca or mixed with a high last-male sperm precedence for fertilization (Reinhardt and Köhler, 1999; Reinhardt, 2000) that may result from higher motility of fresh compared to older sperm (rabbit: Aitherton et al. (1979), chicken: Lodge et al. (1971), sea urchin: Levitan et al. (1991), mussel: André and Lindegarth (1995)) or from differences in sperm longevity (various insects: Hunter and Birkhead (2002), crickets: García-Gonzáles and Simmons (2005)). In contrast, species in which females copulate only once are thought to have reduced costs by minimizing time and energy spent for copulations and reduced risk of predation during vulnerable periods of courtship and mating. Since *Ch. biguttulus* females cycle between no readiness to copulate during 'primary and secondary rejection' and high readiness to mate during 'active copulatory readiness', males should select receptive females for courtship and attempted matings, as it was previously demonstrated in a field study on this species (Kriegbaum and von Helversen, 1992). Indeed, individual females in our laboratory populations received more mating attempts by males on days

when they actively stridulated than on days when they remained mute.

Central nervous and hormonal mechanisms that mediate the different reproductive states and bias females to perform the associated behaviors have not been studied in *Ch. biguttulus* or any other species that also lacks 'passive copulatory readiness' and mates multiply without interspersed ovipositions. 'Primary rejection' seems to be associated with sexual immaturity. *G. rufus* females whose corpora allata were removed during the last nymphal instar or immediately after imaginal molt retained underdeveloped ovaria that produced yolk free oocytes and displayed 'primary rejection' throughout their lives (Loher, 1966). Later studies on this species further implicated juvenile hormone synthesis by the corpora allata in the regulation of reproductive states (Hartmann et al., 1994). 'Secondary rejection', meaning the loss of sexual receptivity after mating, is mediated by a protein from the accessory glands that male *G. rufus* transfer to females during copulation (Hartmann and Loher, 1996). Its stimulation of chemoreceptors in the female spermathecal bulb is usually terminated by the removal of the proteins during the next oviposition. Since *Ch. biguttulus* females re-establish 'active copulatory readiness' without interspersed oviposition, it is unlikely that a similar chemical signal induces 'secondary rejection' in this species. Whether mechanical stimulation by the spermatophore itself may induce the switch to 'secondary rejection' in *Ch. biguttulus* is also questionable since it neither induced rejective states in *G. rufus* (Hartmann and Loher, 1996) nor in *E. brachyptera* and *Ch. curtipennis* which display 'passive copulatory readiness' following mating (Renner, 1952; Hartmann and Loher, 1974, 1996, own unpublished observation). In order to identify the mechanisms that regulate the reproductive states of *Ch. biguttulus* females, our future studies will explore the interplay of central nervous (mediated by the central complex in the protocerebrum) and hormonal (mediated by the secretory systems of the corpora allata and corpora cardiaca) control of reproduction-related female sound production. Its description in the present publication will provide essential information to interpret experimental results of these and other studies that rely on various aspects of the reproduction-related acoustic communication in *Ch. biguttulus*.

3.2 Alteration of female behavior by allatectomy and JH III application

3.2.1 Introduction

As demonstrated by Loher (1962), the behavior of female *G. rufus* changes after removal of the CA (allatectomy). When he removed the CA in the last larval state or shortly after imaginal molt, the adult females did not copulate with males and never reached 'active readiness'. They remained rejective and defended male mating attempts for their entire lives. Reproductive behavior of alltectomized females reappeared after implantation of CA from females in the state of active readiness. Since JH is produced in the CA, it was suggested that the effects Loher observed in allatectomized females were due to the lack of JH. Indeed, Smith and Schal (1990) could show that application of a JH-analog can rescue the effects of allatectomy in cockroach females where dissection of the CA impairs the production of pheromones.

Allatectomy cannot only be achieved surgically but also chemically with precocenes (Pener et al., 1978). Chemical allatectomy could be preferable, because it is less intervening and less stressful for the animal than dissection of the CA. Precocenes are secondary plant metabolites, first isolated from *Ageratum houstianum* and described by Staal (1986). They specifically damage CA tissue after being oxidized to a highly reactive epoxide by enzymes involved in the formation of JH (Pratt et al., 1980; Staal, 1986). Not every insect order is equally sensitive to precocene. In some species, it has a reversible effect on JH synthesis without atrophy of the CA. Others are very sensitive and react with complete, irreversible degeneration of the CA (Staal, 1986). Orthopteran species like *Locusta migratoria* and *Schistocerca gregaria* show high sensitivity to precocene (Schooneveld, 1979a; Fridman-Cohen et al., 1984).

In many studies, the insects were treated topically with precocene dissolved in acetone (Kruse-Pedersen, 1978; Chênevert et al., 1981; Erzyilmaz et al., 2006), but it is also possible to administer precocenes orally by feeding the animals on *Ageratum houstianum* (Triseleva, 2003). For my studies I decided to use precocene dissolved in acetone for top-

ical treatment of the females used in behavioral experiments to have better control over the amount of precocene a female received. Although precocene has specific effects on the CA tissue, it can also have side effects on other tissues, especially the gut (Triseleva, 2003). It was therefore necessary to find the highest possible concentration of precocene that did not severely impair the female. In a pretest, females were treated topically with different concentrations of precocene I dissolved in 5 μL acetone. The solution was applied to the abdomen. A precocene concentration of 0.1 M turned out to be optimal.

Also JH III can be dissolved in acetone and topically applied to the abdomen (Tawfik et al., 2002), but depending on the study, it is administered in different concentrations and frequencies. Tawfik et al. (2002) apply 150 μg JH on three subsequent days to *Locusta migratoria*. Adjusted to the lower weight, this would correspond to approx. 23 μg for a *Ch. biguttulus* female. However, in a pretest, a triple application of 23 μg JH III dissolved in acetone was lethal to the animals and it was decided to treat them only once with 23 μg JH III dissolved in 5 μL acetone.

The behavioral experiments were conducted with three control and three treated females grouped with six males. In the first experimental series, females underwent chemical allatectomy with precocene I dissolved in acetone directly after imaginal molt. The females of the control group received only acetone. In a rescue experiment, females of the treatment group first received the precocene/acetone solution and then, on the next day, an acetone/JH III solution, containing 23 μg JH III. In this experimental series, control females received two times 5 μL acetone.

The experiments were conducted to explore potential changes in the stridulatory and mating behavior of the females and potentially different preferences of males for allatectomized or control females. To check for the effects of precocene on the CA, a pre-experiment with females either fed on *Ageratum houstianum* or treated topically with precocene/acetone solution was conducted and the integrity of their CA tissue was compared to a control group treated only with acetone.

3.2.2 Materials and methods

Group experiment with allatectomized females

Before the experiment, females were randomly assigned to the treatment or the control group. In the treatment group, 5 μL of a 0.1 M precocene I (Aldrich)/acetone solution were applied topically to the ventral abdomen 0-1 days after the imaginal molt. Females in the control group received 5 μL acetone. For discrimination, the females were individually marked with colors (Lukas CRYL TERTIA).

Six males that had been isolated for at least three days, three control females, and three precocene treated females, one to two days after imaginal molt, were brought into a wire woven cage with a base area of 12.5 $\times$ 9.5 cm and a height of 5.5 cm. The cage area was heated to constant 30°C with a heat lamp and additionally illuminated with two light bulbs. The behavior of the animals in the cage was videotaped with a digital video camcorder (MV10, Canon) for 62 min and reviewed on a monitor (HR Trinitron, SONY). The experiments were conducted every day between 1 pm and 3 pm until every female exceeded an age of 12 days after imaginal molt. Females that died during the experiment were replaced by younger ones that had received the same treatment. In the videos, different features of the grasshoppers' behavior were identified. Mating attempts by the males were identified, if the typical mating sound, described by (Faber, 1929), had been produced while the male was directed toward the female. In addition, mounting of females by the males and the stridulatory activity of the females were evaluated. Mountings were regarded as matings if they lasted longer than two minutes. The results for mating attempts, matings, and stridulation were analyzed with Excel (Microsoft). The experiment was run four times with different grasshopper females. After checking for the presence of CA tissue in different precocene treated females in a parallel experiment, it turned out later that the precocene or acetone treatment had to be carried out no later than 1 day after imaginal molt to successfully destroy the CA, 2 females of each group had to be excluded from the analysis. After the experiments, precocene treated females were examined to check for the presence of CA.

Rescue

In this experimental series, females of the treatment group received 5 μL of the precocene/acetone solution 0-1 day after imaginal molt and 5 μL of acetone containing 23 μg JH III (MP Biomedicals) topically applied to the ventral abdomen on the following day. Females of the control group received two treatments with acetone on the same days after imaginal molt. Three experimental series with different grasshopper females were conducted. Due to the fact that two females escaped and encountered males outside of the observation cage, only data of eight females in every group were analyzed.

Statistics for group experiments

Every female was tested from day three to day twelve after imaginal molt. Within observation periods on each day, the mating attempts directed toward a specific female were counted. For each observation period, the total number of stridulating females in the experimental cage was determined and it was noted, whether the particular female itself was stridulating. Data were analyzed in a generalized estimating equations (GEE) model. The data were fitted to a negative binomial distribution with a log-link function. 'Behavior' (female is stridulating: yes = 1 / no = 0) and 'Treatment' (treatment group = 1, control group = 0) were defined as factors in the model, 'Age' (day after the female's imaginal molt) and 'Singers' (number of stridulating females in the cage) as covariates. In addition, it was tested for interaction effects of 'Behavior' with 'Treatment', 'Age', or 'Singers'. Female identity nested within an experimental group was entered as a subject effect. For the within subject factor 'Age', a first order autoregressive working correlation matrix was chosen. The effects of factors and covariates on the number of male mating attempts were assessed.

Controlled matings with subsequent JH treatment

Isolated virgin females were set into a 15 × 12 × 12 cm acoustically isolated test chamber and exposed to three recorded sequences of a male calling song that were played five times with an interval of five seconds via a loudspeaker (Sound Craft Conrad DT 25 P). The

speaker was positioned at a distance of 10 cm to the cage and its volume was adjusted to 65 dB/SPL. The temperature was constant at 30°C. Responding to this calling song by stridulation was regarded as a measure for high copulatory readiness of the female. If the female responded to the song, three intact male *Ch. biguttulus* were set into the same cage. Usually between 1 and 30 minutes, one of the males started copulating with the female. Matings were assessed as successful, if two criteria were fulfilled: 1) A firm connection between the sexual organs was established, 2) this connection lasted for more than two minutes. Females whose matings did not match one or both of the criteria were excluded from further analysis. Twelve hours after mating, the females received a dose of 23 μg JH III dissolved in 5 μL acetone that was applied topically to the abdomen. A control group received 5 μL acetone. On the following 14 days, the responsiveness of the mated female to the presentation of a male calling song was tested. The complete experiment was performed with each of 13 treated and control females.

Electron microscopy of CA tissue

Ageratum houstianum plants were raised from commercially obtainable seeds. Females of the 'Ageratum-group' were set into a cage with one plant as the sole food source. Control females were maintained at their regular diet. After 24 hours, feeding tracks were noticeable at the leaves, and the females' CA were dissected for electron microscopy. Females in the 'Control-group' and the 'Precocene-group' were either treated topically with 5 μL acetone or 5 μL 0.1 M precocene (Aldrich)/acetone solution, respectively. The CA were dissected after 24 hours.

The tissues of all females were fixed with 4% paraformaldehyde (Serva) and 0.5% glutaraldehyde (Serva) in 0.1 M PO_4 buffer at pH 7.4 for 3 hours at 4°C. After rinsing in 0.1 M PO_4 buffer two times for five minutes at room temperature, 2% OsO_4 (TAAB Laboratories Equipment Ltd) in 0.1 M PO_4 buffer was applied for 1 hour at 4°C. The tissue was rinsed shortly in 0.1 M PO_4 buffer and dehydrated with an ethanol (Merck) series (30%, 40%, 50%, 60%) each concentration two times for 7 minutes. A contrast enhancing step with uranylacetate was conducted for 1 hour in 70% EtOH. Afterwards,

the ethanol series was continued until 100% EtOH, each step two times for 10 minutes, before the specimen were transfered to propylenoxid (Merck). They were then incubated in a 3:1 mixture of propylenoxide/araldite (Serva) for 1 hour, 1:1 propylenoxide/araldite for 12 hours, and 1:3 propylenoxide/araldite for 1 hour at room temperature before they were transfered to blocks filled with pure araldite and heated to 60°C for 48 hours to solidify. From the hardened araldite blocks, 1 μm sections were cut with an ultramicrotome (Reichert-Jung, ULTRACUT E) and stained with toluidinblue (Merck) for 5 minutes. Microphotographs were taken with a transmission electron microscope (ZEISS, EM 902A). Cell organelles and other cellular components were identified after Schooneveld (1979b), Piulachs et al. (1989), and Johnson et al. (1993).

3.2.3 Results

Group experiment with allatectomized females

Data of ten treated and ten control females were analyzed. Until day 12 after imaginal molt, 90% of the control females but only 30% of the treated females had stridulated at least once. In comparison with the experiment with females that received no treatment at all (see chapter 3.1), females of the acetone treated control group started to stridulate relatively late ($\tilde{X}$ = 9.56 days, IR: 9-12 days, in acetone treated females compared to 5.9 days in untreated females). Precocene treated females started to stridulate with a median of $\tilde{X}$ = 12 days, IR: 11-12 days. After testing for equal distributions around the median with a Mann-Whitney U test ($U_{9;3}$ = 15.5, corrected for ties: $z(U)$ = 0.385, N_{ctrl} = 9, N_{treat} = 3, P = 0.351), a Mann-Withney U-test to compare the medians was applied. The difference in the days of first stridulation between acetone only and precocene treated females was not significant ($U_{9;3}$ = 8, corrected for ties: $z(U)$ = 1.0655, N_{ctrl} = 9, N_{treat} = 3, P = 0.144, *95% CI*: -7, 0.9).

The number of male mating attempts directed toward singing and silent treated and control females was analyzed in a GEE model (Fig. 7), fitted for negative binomial distribution. In this experiment, neither the treatment (GEE: χ^2_1 = 0.086, P = 0.769) nor female stridulatory behavior (GEE: χ^2_1 = 2.162, P = 0.141) had a significant influence

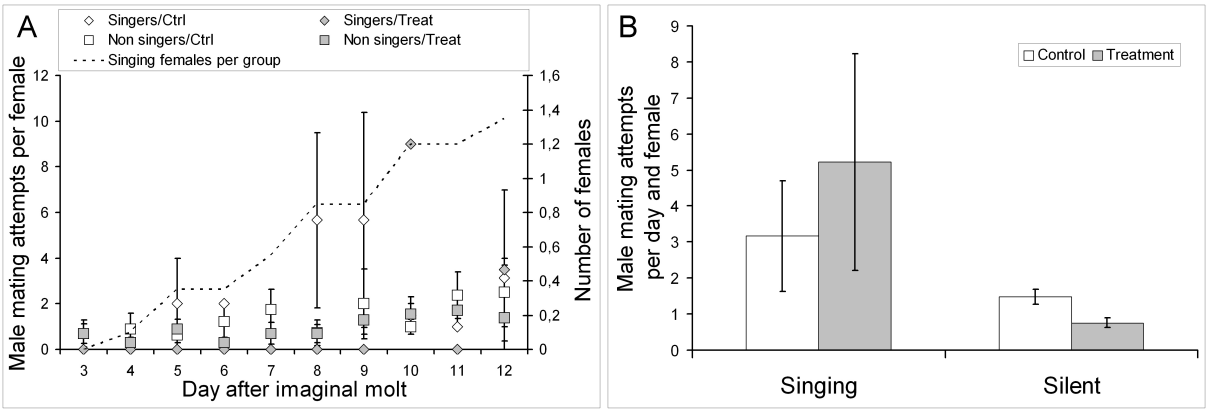

Figure 7: *Male mating attempts toward singing and silent females in small laboratory populations after treatment with precocene I*
A: Average number of mating attempts directed toward singing and non singing females of control (Ctrl) and treatment (Treat) group (left Y-axis) in relation to singing females per group at a particular female age (background stridulation, right Y-axis). Error bars SEM. Analysis by GEE revealed no influence of female age, treatment, behavior, or background stridulation on male mating attempts. **B:** Average number of mating attempts received by individual females. Although singing females received more mating attempts than silent females, the difference was not significant. There was no male preference for treated or control females detectable. Error bars: SEM.

on the number of male mating attempts. Allatectomized females received on average 0.18 mating attempts more than control females (*95% CI*: -1.02, 1.37). Singing females received on average 3.00 mating attempts more than silent females (*95% CI*: -0.70, 6.71). An interaction effect of 'Behavior' and 'Treatment' was also not detectable ($\chi^2_1 = 1.582$, $P = 0.208$).

Only three out of ten precocene treated females started to stridulate during the observation time of 12 days after imaginal molt, while nine of the ten acetone treated females stridulated until day 12. Figure 8A shows the percentages of females that stridulated at least once until a certain day after imaginal molt in the control and in the treatment group. A Fisher's exact test revealed that the difference in number of stridulating females becomes significant for day 11 and 12 (day 11: $F_{4;12} = 4.385$, $N_{ctrl} = 10$, $N_{treated} = 10$, $P = 0.029$; day 12: $F_{8;18} = 3.825$, $N_{ctrl} = 10$, $N_{treated} = 10$, $P = 0.010$). After the behavioral experiments, all females were examined for the presence of CA. In all control females, CA were present. Among the precocene treated females, CA were found in three animals, two of them started to stridulate during the experiment.

Despite the incomplete chemical allatectomy in three of ten precocene treated females, none of these females mated. In contrast, 50% of the females in the control group mated

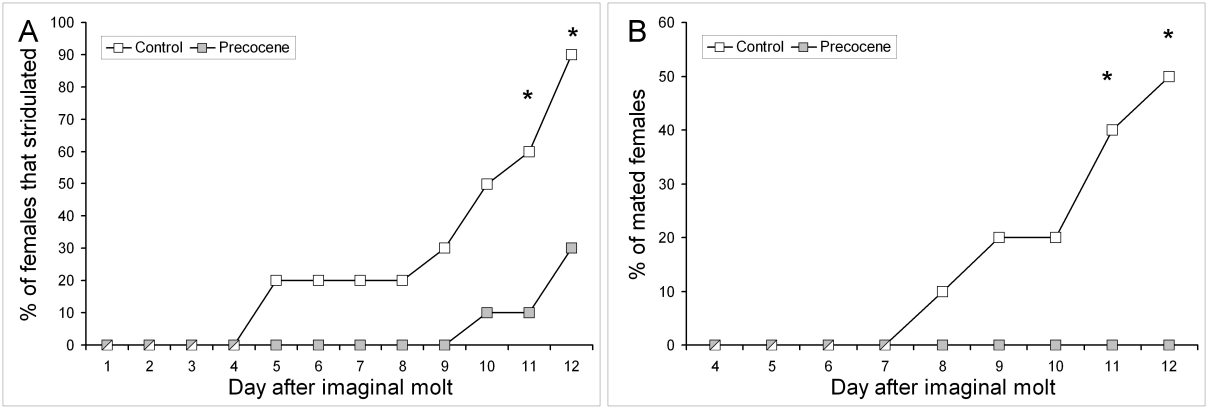

Figure 8: *Percentages of stridulating and mated females in control and allatectomized females*
A: Percentage of females that had stridulated at least once in the control group compared to the precocene treated group. The difference between the groups is significant on day 11 and 12 after imaginal molt. **B:** Percentage of females that have copulated (mated) at least once in the control group compared to the precocene treated group. The difference between the groups is significant for day 11 and 12 after imaginal molt.

until day 12 after imaginal molt (Fig. 8B). The difference is significant on day 11 and 12 (Fisher's exact test: day 11: $F_{2;8} = 5.33$, $N_{ctrl} = 10$, $N_{treated} = 10$, $P = 0.043$; day 12: $F_{2;10}$, $N_{ctrl} = 10$, $N_{treated} = 10$, $P = 0.016$).

Rescue

In this experiment, females of the treatment group received precocene I on day 0-1 after imaginal molt and JH III on the following day. Both, precocene and JH III, were applied as dilutions with 5 μL acetone. Control Females received two times 5 μL acetone applied topically on the abdomen. In both groups, 75% of the females started to stridulate until day 12 after imaginal molt. Control females started to stridulate with a median of $\widetilde{X} =$ 8 days, interquartile range: 6-10 days, treated females started to stridulate with $\widetilde{X} =$ 7.5 days (IR: 6-9 days). Although, precocene/JH treated females started to stridulate a little earlier, there was no significant difference between the two groups. After testing for equal variances with a Mann-Whitney U-test ($U_{6;6} = 17$, $N_{ctrl} = 6$, $N_{treated} = 6$, $P >$ 0.05), the median days of first stridulation were compared with a Mann-Whitney U test ($U_{6;6} = 13.5$, corrected for ties: $z(U) = 0.7403$, $N_{ctrl} = 6$, $N_{treated} = 6$, $P = 0.230$, *95% CI*: -2.9, 4).

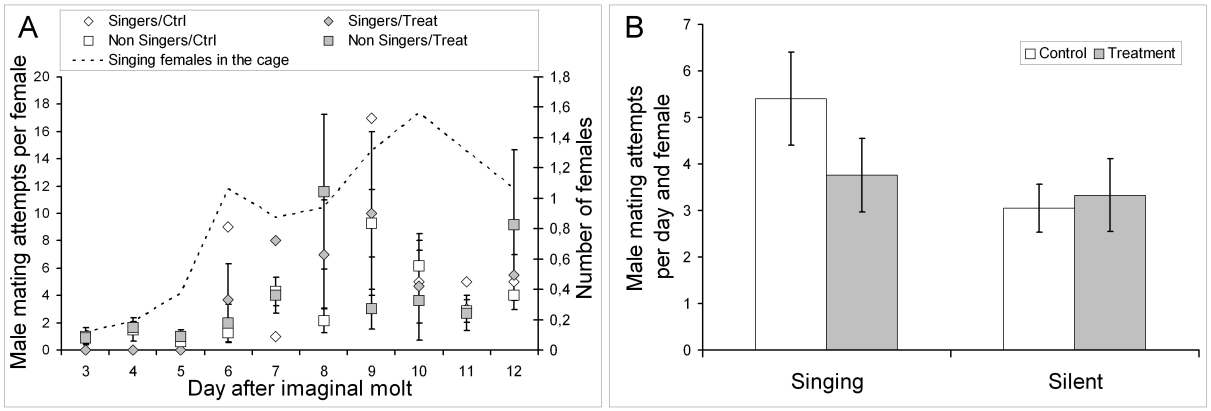

Figure 9: *Male mating attempts toward singing and silent females in small laboratory populations after treatment with precocene I and JH III*
A: Average number of mating attempts directed toward singing and non singing females of control (Ctrl) and treatment (Treat) group (left Y-axis) in relation to singing females per group at a particular female age (background stridulation, right Y-axis). Error bars SEM. Analysis by GEE revealed no influence of female age, treatment, behavior, or background stridulation on male mating attempts. An interaction effect between 'Behavior' and 'Age' was present. Older non singers received more mating attempts than younger non singers. **B:** Average number of mating attempts received by individual females. Although singing females received more mating attempts than silent females, the difference was not significant. A male preference for treated or control females was not detectable. Error bars: SEM.

According to the GEE model, the factors 'Treatment' and 'Behavior' had no significant influence on male mating attempts (Treatment: $\chi^2_1 = 0.454$, $P = 0.501$, Behavior: $\chi^2_1 = 3.561$, $P = 0.059$). Precocene/JH treated females received on average 0.52 mating attempts less than control females (*95% CI*: -1.80, 0.75), Singing females received on average 1.32 more mating attempts than silent females (*95% CI*: -0.37, 3.00). The numbers of mating attempts in the rescue experiment are depicted in Fig. 9.

In this experiment, an interaction effect of 'Behavior' and 'Age' was observed (GEE: $\chi^2_1 = 4.807$, $P = 0.028$). With increasing age, silent females received $X + SE = 1.25 + 0.24$ mating attempts more than the day before.

Control and treatment group contained equal numbers of females that started to stridulate within 12 days after imaginal molt and there was no difference in the timepoint when females began to stridulate (Fig. 10A). In contrast, mating activity of the two groups appeared to be different. Although (probably due to the low number of tested animals), the difference was not big enough to be significant (Fisher's exact test, data not shown). It was remarkable that only one out of eight treated females but five out of eight control females mated (Fig. 10). In none of the precocene/JH treated females, CA

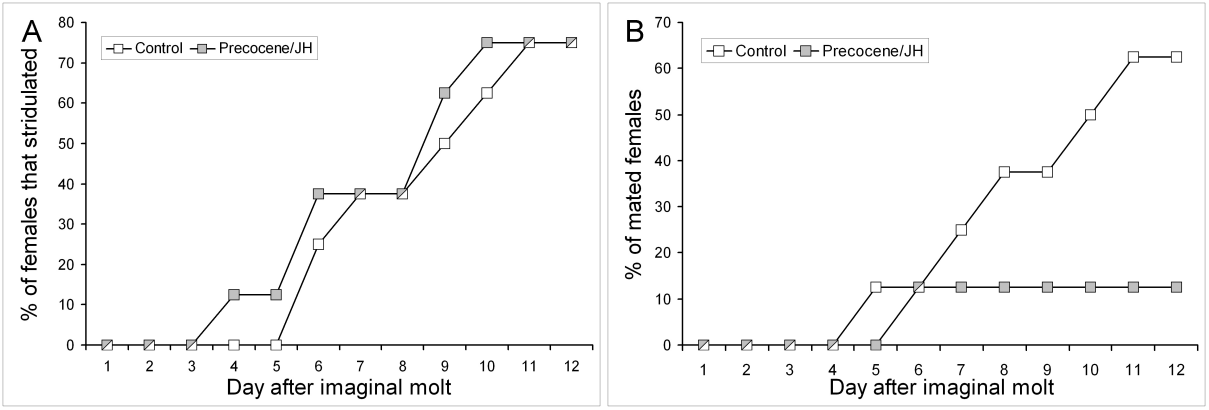

Figure 10: *Percentage of stridulating and mated females in control and treatment group*
A: Percentage of females that had stridulated at least once in the control and in the precocene/JH treated group. In both groups, 75% of the females stridulated until day 12 after imaginal molt. A difference between the groups could not be detected. **B:** Percentage of females that had copulated (mated) at least once until day 12 after imaginal molt. Only one of eight females in the treatment group mated, in comparison to five out of eight females in the control group. Nevertheless, the difference was not significant.

were detected during the examination after the behavioral experiment.

Controlled matings with subsequent JH treatment

Females that had responded to the record of a male calling song were allowed to mate with males and 12 hours later were treated with JH III (treatment group) or acetone only (control group). Within the experimental period, 12 out of 13 females in the control group started to stridulate again. In the treatment group, 11 out of 13 females restarted stridulation (Fig. 11A). A D'Agostino Pearson omnibus test detected no deviations from normal distribution for the days when stridulation restarted ($K^2_{ctrl} = 2.32$; $K^2_{treat} = 0.91$). After testing for equal variances with an F test ($F_{11;10} = 0.54$; $P = 0.34$), a two-tailed t-test for samples with equal variances was applied ($t_{21} = 0.83$, $P = 0.42$, *95% CI*: -1.95, 4.54 days). Although, females that received JH started to stridulate 1.3 days earlier after mating, a difference in the duration of secondary rejection could not be shown (Fig. 11B).

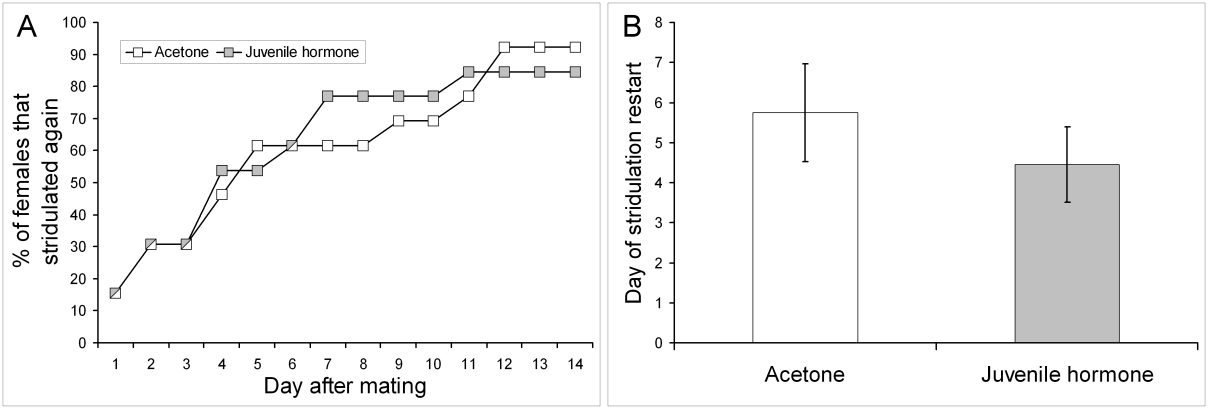

Figure 11: *Secondary rejection after JH treatment*
Females were topically treated with JH III dissolved in acetone 12 hours after copulation with a male. **A:** Equal amounts of females restarted to stridulate within the experimental period of 14 days. **B:** In both groups, stridulation restart occured relatively late after mating when compared to untreated females. JH treated females started to stridulate earlier than acetone treated females but the difference was not significant. Error bars: SEM.

Electron microscopy of CA tissue

In comparison to the control group, the CA tissue from females either fed on *Ageratum houstianum* or treated with precocene I showed signs of degeneration or inactivation (Fig. 12). In the CA tissue of precocene treated females (Fig. 12B), the immigration of haemocytes could be observed, nuclear membranes were invaginated, and condensed cell fragments were present that were previously interpreted as the final stage of autophagocytosis (Cheng and Chiang, 1995). In the CA tissue of *Ageratum houstianum* fed females (Fig. 12C), those effects were even more pronounced. Also signs for inactivation of the CA tissue could be identified after Dai and Gilbert (1991), like whorls of smooth ER and cells of symbiontic microorganisms (Fig.'s 12E and D).

3.2.4 Discussion

Precocene I was used for chemical allatectomy in the behavioral experiments. Since precocenes might interfere with enzymes involved in JH synthesis, there is the assumption that their impact on CA tissue degeneration is stronger during phases of high JH synthesizing activity (Staal, 1986, review). Although this influence could be demonstrated only once (Unnithan and Nair, 1979), precocene was applied to grasshopper females immediately after imaginal molt, when CA activity is presumably higher than later in life

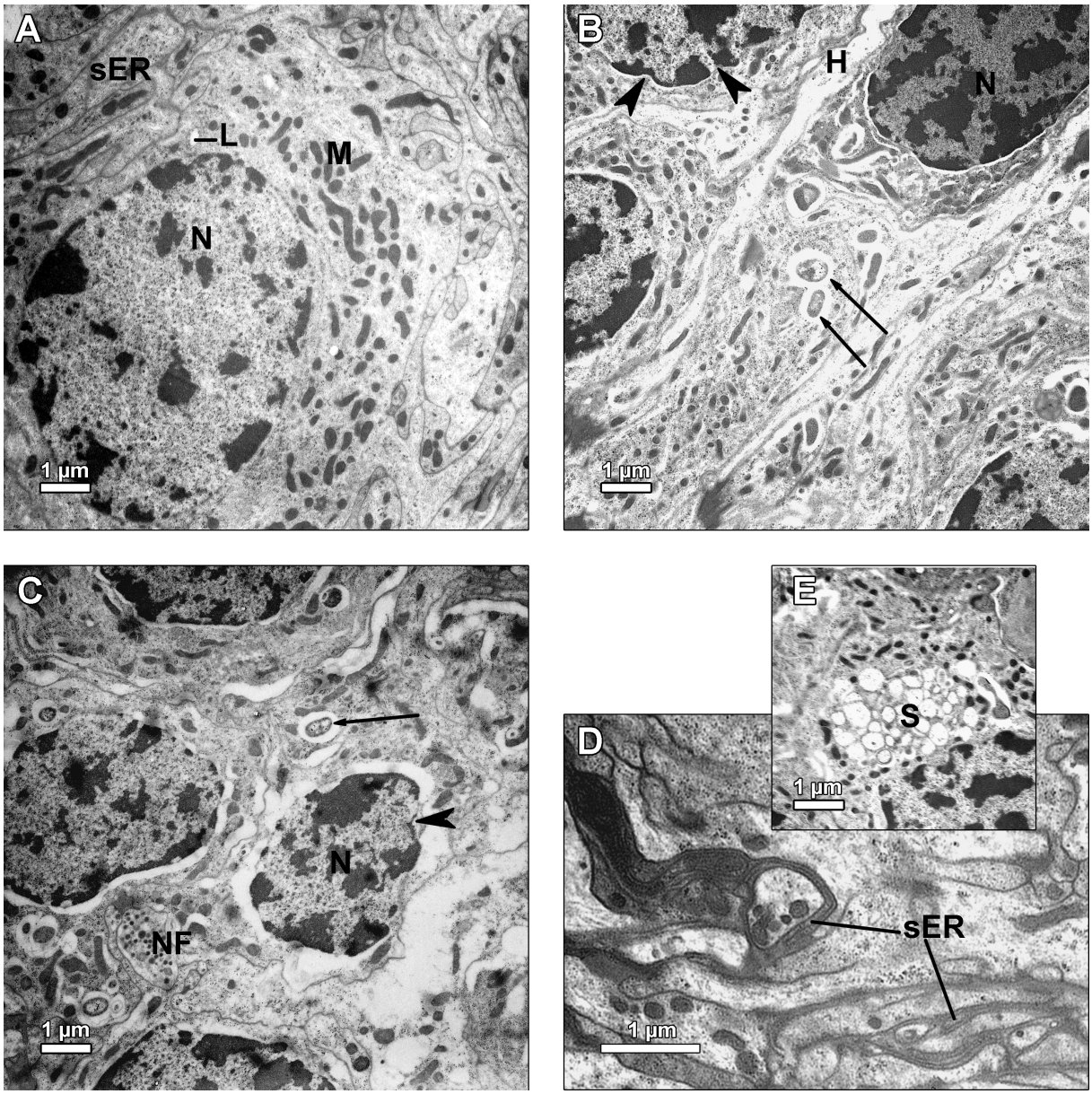

Figure 12: *Microphotographs of CA tissue after treatment with precocene I and feeding on* Ageratum houstianum

A: Control, treated with acetone, no invaginations of the nucleus (N), none of the preparations contained condensed cell fragments. **B:** CA tissue 24 hours after precocene treatment. Haemocytes (H) have migrated into the tissue, invaginations of the nuclear membrane can be found (arrow heads), and many condensed cell fragments (thin arrows). **C:** CA 24 hours after feeding on *Ageratum houstianum*. Again, many condensed cell fragments and invaginated nuclear membrane. **D+E:** Details of CA tissue after precocene treatment. **D:** Whorls of smooth ER (sER). **E:** Presumably cells of symbiontic microorganisms (S), either Rickettsia or Mykoplasma. Further abbreviations: T: Trachea, M: Mitochondria, L: Lipid droplet, NF: neurosecretory fiber.

(Hartmann et al., 1994).

As previously reported for other insect species like *Locusta migratoria* (Schooneveld, 1979b), *Blattella germanica* (Piulachs et al., 1989), and *Anacridium aegyptium* (Ergen, 2001), precocene treatment leads to a macro- and microscopically visible degeneration of CA tissue in *Ch. biguttulus* females. Typical signs of degeneration and inactivation were observed 24 hours after topical treatment with precocene dissolved in acetone or after feeding on *Ageratum houstianum*. Nuclear membrane invaginations, immigration of haemocytes, and condensed cell fragments were observed as well as whorls of smooth endoplasmatic reticulum and cells of symbiontic microorganisms that represent signs of inactivity (Dai and Gilbert, 1991). Surprisingly, grasshopper females fed on *Ageratum* accepted the nutrition with this plant and accumulated enough precocene for severe degeneration of their CA. After 24 hours signs of degeneration in their CA tissue appeared even more prominent than after direct application of precocene I to the abdomen. While first signs of degeneration were observed already 24 hours after precocene application, in 15 out of 18 females that had been treated with precocene I until the first day after imaginal molt, CA were not detectable, indicating that the treatment caused a complete loss of CA tissue.

Treatment with precocene I affected both, the stridulatory and mating behavior of the female grasshoppers. None of the treated females permitted the copulation with a male. Three precocene treated females started to stridulate within the experimental period which was probably due to incomplete degradation of the CA. In two of them, CA tissue was still present.

Application of JH III restored the stridulatory behavior in precocene treated females. Control and precocene/JH III treated group did not differ with respect to the number of animals that stridulated and the time of first stridulation. Together with the observation that precocene induced allatectomy inhibits females stridulation, this indicates that the presence of JH is pivotal for the performance of female stridulation. This was different for the mating behavior which could not be restored by the application of JH III after allatectomy. Only one female of the treatment group permitted the copulation with a

male.

In a number of insects, JH seems to be involved in the control of sex pheromone production (Boriden et al., 1969; Smith and Schal, 1990; Rantala et al., 2003). Interference with pheromonal communication could influence the attractiveness of a female to males and change the overall mating activity of an individual female. In the behavioral experiments, it was therefore checked, if there was a difference in males' preference for either allatectomized or control females. This was not the case. Males directed as many mating attempts toward control females as toward allatectomized females. While the interference of allatectomy with the pheromonal system cannot be excluded for *Ch. biguttulus*, differences in mating activity are not due to a difference in female attractiveness for the males and can only be explained with a rejective behavior of the females.

Why does the application of JH III restore the stridulatory but not the mating behavior of allatectomized females? An explanation might lie in the fast degradation of JH III. In contrast to JH analogs like pyriproxyfen, JH III is degraded from the hemolymph by enzymatic processes. Cornette et al. (2008) applied a dose of 30 μg JH III dissolved in 5 μL acetone to damp-wood termites (*Hodotermopsis sjostedti*) and were able to increase the JH concentration in the hemolymph from 175 pg/μL to more than 8 ng/μL. They did not monitor the degradation of the extra JH III in a time series but found that this high exogenous dose was completely degraded within seven days.

In this study, a similar dose of JH III was administered to allatectomized female *Ch. biguttulus*. Females in the control group mated on average at nine days after imaginal molt. If JH III is equally fast degraded in *Ch. biguttulus* as in *Hodotermopsis sjostedti*, it was expired before the time of first mating. This could lead to a suppression of normal mating behavior which was observed in precocene/JH III treated females. The application of a single dose of JH III to allatectomized grasshopper females shortly after the imaginal molt might be sufficient to evoke ovary maturation and elicit stridulatory behavior, but JH III seems to be necessary to sustain reproductive behavior.

Application of JH III after mating showed the tendency to shorten secondary rejection, although, a significant difference to the control group could not be shown, and stridulation

does not always indicate reproductive readiness. This will be discussed in more detail in chapter 6.1 in combination with the results for JH titer measurements.

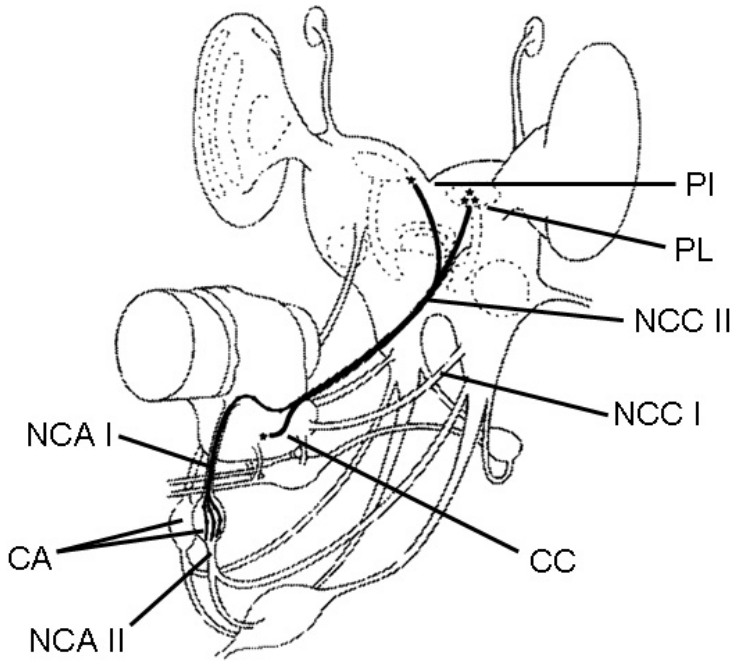

Figure 13: *Anatomical scheme of brain and retrocerebral complex connections, modified after Vullings et al. (1999)*
The corpora allata (CA) receive inputs from neurons in the pars intercerebralis (PI) and the pars lateralis (PL) of the brain. Their axons enter the nervi corporis cardiaci I and II (NCC I and NCC II, pass the corpora cardiaca (CC) and reach the CA via the nervus corporis allati I (NCA I). Other inputs come from the subesophageal ganglion that enter the CA via the nervus corporis allati II (NCA II).

4 Immunocytochemistry

4.1 Introduction

The corpora allata are supplied by two nerves, the nervus corporis allati I and the nervus corporis allati II (NCA I and NCA II) (Fig. 13). The NCA I connects the corpora allata with the corpora cardiaca (CC), a pair of neuroendocrine glands building the retrocerebral complex together with the CA. Via the nervus corporis cardiaci I (NCC I) and the nervus corporis caridaci II (NCC II), cells from the pars lateralis (PL) and from the pars intercerebralis (PI) project directly into the CA. Others are interconnected in the CC (Mason, 1973). By backfills of the NCA I, Virant-Doberlet et al. (1994) found neurons in the ipsilateral and contralateral PL that projected to the corpus allatum via both, the ipsilateral and the contralateral NCC II. Some of the neurons innervated only one, others both CA. In addition, there are somata in the tritocerebrum sending axons through the

nervus corporis cardiaci III and the NCA II that pass the CA and branch in the CC (Vullings et al., 1999). But fibers in the CA are not only derived from brain neurons. Via the NCA II e.g. CCAP-immunoreactive somata in the subesophageal ganglion project to the retrocerebral complex (Vullings et al., 1999) and the CC themselves contain intrinsic neurosecretory cells that branch in the CA (Copenhaver and Truman, 1986).

NADPHd activity in the CA could be detected in many orders like Lepidoptera (Zayas et al., 2000, *Manduca sexta*), Blattodea (Chiang et al., 2000, *Diploptera punctata*), and Diptera (Wildemann and Bicker, 1999, *Drosophila melanogaster*). In the locust *Schistocerca gregaria*, (Kurylas et al., 2005) found NOS immunoreactive fibers in the median bundle, two fiber tracts that contain, amongst others, axons of PI neurons projecting to the corpora allata.

It seems likely that NOS immunoreactive fibers in the CA originate in the brain. Stainings with an antibody against citrulline, the byproduct of NO-generation, showed intensely stained cell bodies in the PI (Weinrich et al., 2008) where fibers that innervate the CA have their origin, too (Virant-Doberlet et al., 1994). To reconstruct the neuroanatomy of NO producing structures in the retrocerebral complex, either the CC or the CA were backfilled with neuronal tracers. These preparations were subjected to immunocytochemistry against NOS or citrulline. This intended to reveal whether the NO generating activity found in the retrocerebral complex could derive from cells in the PI. Neurobiotin was used as neuroanatomical tracer. It is a relatively small molecule that, once introduced to the cell, is distributed into every process by intracellular transport mechanisms. Neurobiotin has a high affinity to avidin and can be detected with fluorescent dyes coupled to streptavidin or avidin (Köbbert et al., 2000), and thus enables analysis of labeled preparations by confocal microscopy. In addition, backfills were combined with immunocytochemistry against cGMP, allatotropin, allatostatin and other neurotransmitters to explore projections and properties of retrocerebral cells.

Skinner et al. (2000) found NOS immunoreactive fibers in the CC in close proximity to allatostatin immunoreactive fibers in *Diploptera punctata* which suggested an influence of NO on the release of allatostatin. For NOS immunoreactive neurons in the brain, Kurylas

et al. (2005) presumes similarities to neurons detected by immunostainings against allatotropin (Homberg et al., 2004) and -statin (Vitzthum et al., 1996), but there have been no attempts to find colocalizations so far.

4.2 Material and methods

Animals

Studies were performed with female nightingale grasshoppers of the species *Chorthippus biguttulus* and female pacific beetle cockroaches (*Diploptera punctata*).

Most of the grasshopper females were reared from eggs that had been collected during the previous summer and kept at 4°C for at least four months. After approximately one week at 26°C, the nymphs hatched and were fed with grass (*Poa spec.* and *Dactylis spec.*) and supplemental food for crickets (Nekton, Pforzheim, Germany) ad libitum.

Cockroaches were purchased from J. Bernhardt (www.schaben-spinnen.de). Until usage, they were kept in a 55 × 35 × 27 cm box at 24°C and fed with potatoes and fruits.

Dissection and general immunocytochemical procedure

To prevent battology, the common steps of all immunocytochemical experiments are described. For particular stainings only deviations and additions of this protocol will be described afterwards.

Animals were anesthetized by cooling to 4°C. Subsequently, they were fixed to a Plasticine (Pelikan) bed and the head was opened dorsally. After removal of tracheas and covering fatty tissue, the brain was dissected. For fixation, brains were transferred into a solution containing 4% paraformaldehyde in 0.1 M PO_4 buffer and incubated for two hours at 10°C. Embedding in gelatine albumine and postfixation in the same fixative were followed by cutting the brains into 50 μm thick sections with a vibratome (LEICA, VT100S). After permeabilization of the tissue overnight with Triton X-100 (SIGMA) dissolved in PBS, the sections were incubated in blocking buffer consisting of PBS containing normal donkey serum (Jackson ImmunoRe-

search) or normal goat serum (Amersham Biosciences), bovine serum albumine (MP Biomedicals, Inc.) and Triton X-100. Blocking with sera aims to saturate possible unspecific binding sites for secondary antibodies in the tissue. Primary antibodies were applied in blocking buffer for at least 24 hours. Afterwards, the primary antibodies were removed by several rinses in PBST. Secondary antibodies were as well applied in blocking buffer for 3 hours at a concentration of 1:300. The sections were rinsed with PBST two times for 10 min and three times for 10 min with PBS. Thereafter, the sections were mounted on microscopic slides with PBS/glycerol (SIGMA).
Antibodies against uNOS, citrulline, cGMP, allatotropin, allatostatin, serotonin, dopamine, CCAP, and FMRFamide were used after neurobiotin tracing or in combination to detect colocalized components of the NO/cGMP system and the JH control system. Table 2 provides an overview of the used antibodies and their concentrations.

Tracing of CA/CC − brain connections

Animals were anesthetized by cooling to 4°C. Subsequently, they were fixed to a Plasticine bed and the head was opened dorsally. After removal of tracheas and fatty tissue covering the brain, the right corpus allatum was cut and the open end sucked into a capillary, filled with 4% neurobiotin in Aqua dest. The capillary was fastened in its position and the open head was covered with petrolatum (GOETHE-APOTHEKE, Göttingen) to prevent dessication.

To stain possible projections from CA cells into the brain, the brain was incised below the right mushroombody calyx to transect fibers in the pars intercerebralis and lateralis. A dextrane-biotin crystal (Invitrogen) was placed in the incision. The brain was then covered with Locusta saline (Clements and May, 1974) (see Appendix) and petrolatum.

To allow the uptake and distribution of neurobiotin or dextrane-neurobiotin by truncated fibers, the Plasticine bed with the animal was placed into a humid box and cooled for 12-24 hours. After this incubation step in a humid atmosphere, the brains were removed and further processed for immunocytochemistry. Neurobiotin was labeled by the application of a fluorophore-coupled streptavidin.

Citrulline immunocytochemistry

The antibody against the aminoacid citrulline is directed against a citulline-glutaraldehyde conjugate (Martinelli et al., 2002). In contrast to other staining procedures, the tissue fixative has to contain glutaraldehyde to enable a specific binding of the antibody. The fixative consisted of 4% paraformaldehyde and 0.25% glutaraldehyde dissolved in 0.1 M PO_4 buffer. As a consequence of glutaraldehyde fixation, autofluorescence of the tissue emerged. Autofluorescence was supperessed by incubation of the brain sections in 10% sodiumborohydrate (SIGMA) for 10 minutes.

Suppression of NO production with aminoguanidine

Female grasshoppers were injected either with 30 μL of 10^{-2} M aminoguanidine hemisulfate (AG) (SIGMA) dissolved in Locusta saline or, as a control, with 30 μL Locusta saline into the abdomen. Four hours after this treatment, the brains and retrocerebral complexes were removed and processed for citrulline immunocytochemistry.

cGMP accumulation

Brains were dissected on ice and incubated in Locusta saline containing 5×10^{-4} M YC-1 (SIGMA), an activator of soluble guanylate cyclase, for 45 min on ice. They were then transferred to a saline solution containing 5×10^{-4} M YC-1, 10^{-3} M SNP (SIGMA), an NO donor, and 10^{-3} M Zaprinast (SIGMA), a phosphodiesterase inhibitor, and incubated for 45 min at 33°C to accumulate cGMP in the cells. The brains were afterwards fixed and prepared for further immunocytochemistry as described.

cGMP accumulation without SNP

Female *Ch. biguttulus* were injected with 30 μL of an aminoguanidine solution (10^{-2} M aminoguanidine hemisulfate in Locusta saline) four hours before dissection of their brains. cGMP accumulation was conducted as described above with the difference that part of the brains received only YC-1 and Zaprinast but no SNP.

NADPH diaphorase histochemistry

The protocol was adapted from Ott and Elphick (2003). Brains were dissected and fixed in methanol/formalin (91% methanol (MERCK), 3.7% formaldehyde (MERCK)) for 2 hours. They were rinsed 2 × 15 minutes with Tris-buffer (0.1 M, pH 7.2) and then kept in sodiumacetate (MERCK) buffer (0.1 M, pH 4.0) for 1 hour. The brains were incubated on ice for 12 hours in 0.1 M Sørensen phosphate buffer (0.1 M, pH 5.0) containing 0.2 mM β-NADPH (MERCK), 0.2 mM nitroblue tetrazolium (NBT) (SIGMA), and 0.2% Triton X-100. After this treatment, the tissue was placed in staining solution (0.2 mM β-NADPH, 0.2 mM NBT in Tris-TX) at pH 8.0 for 3 hours at room temperature. The staining reaction was stopped by rinsing in Aqua dest. and the brains were transferred into a freshly prepared mixture (1:3) of absolute methanol and acetic acid (MERCK) and incubated for 30 minutes. After three washes à 30 minutes in absolute methanol, the tissue was cleared for 1 hour in cedar oil (CHROMA-GESELLSCHAFT, Stuttgart-Untertürckheim). Eventually, the brains were mounted on cavity slides in cedar oil.

Dopamine staining

Similar to the antibody against citrulline, also the dopamine antibody detects a conjugate from dopamine and glutaraldehyde. The tissue was therefore fixed with 1.5% glutaraldehyde in 0.1 M phosphate buffer. To prevent oxidation of dopamine, sodium metabisulfite (MERCK) was added at a concentration of 10 g/L to the fixative and at 8.5 g/L to all other buffer solutions.

Table 2: *Buffers and antibody concentrations for immunocytochemistry*

Antigens	**Fixative**	**Blocking solution (BS)**	**1st antibodies**	**2nd antibodies**
Allatostatin + uNOS	4% PFA in 0.1M PO_4 buffer	5% NGS, 0.25% BSA, 1% Triton X-100 in PBS	α-Dip-Ast 7 from mouse (DSHB) 1:20, α-uNOS from rabbit (ABR) 1:100 in BS	α-mouse Alexa488 from goat (Molecular Probes) 1:300, α-rabbit Alexa633 goat (Molecular Probes) 1:300 in BS
Allatostatin + cGMP	4% PFA in 0.1M PO_4 buffer	5% NDS, 1% Triton X-100 in PBS	α-Dip-Ast 7 from mouse 1:20, α-cGMP from sheep (J deVente) 1:1000 in BS	α-mouse Cy2 from donkey (Jackson Immunoresearch) 1:300, α-sheep Cy5 from donkey (Jackson Immunoresearch) 1:300 in BS
Allatostatin + neurobiotin	4% PFA in 0.1M PO_4 buffer	5% NGS, 0.25% BSA, 1% Triton X-100 in PBS	α-Dip-Ast 7 from mouse 1:20 in BS	α-mouse Cy5 from goat (Jackson Immunoresearch) 1:300, Cy2 streptavidin (Rockland) 1:300 in BS
Allatotropin + citrulline	4% PFA, 0.25% GA in 0.1M PO_4 buffer	10% NGS, 0.25% BSA, 10% Triton X-100 in PBS	α-Mas-allatotropin from rabbit (D Nässel) 1:1000, α-citrulline from mouse (G Martinelli) 1:20 in BS	α-rabbit Alexa633 from goat 1:300, α-mouse Alexa488 from goat 1:300 in BS

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Antigens	**Fixative**	**Blocking solution (BS)**	**1st antibodies**	**2nd antibodies**
Allatotropin + cGMP	4% PFA in 0.1M PO_4 buffer	5% NDS, 1% Triton X-100 in PBS	α-Mas-allatotropin from rabbit 1:1000, α-cGMP from sheep 1:1000 in BS	α-rabbit Cy2 from donkey (Jackson Immunoresearch) 1:300, α-sheep Cy5 from donkey 1:300 in BS
Allatotropin + neurobiotin	4% PFA in 0.1M PO_4 buffer	5% NGS, 0.25% BSA, 10% Triton X-100 in PBS	α-Mas-allatotropin from rabbit 1:1000 in BS	α-rabbit Alexa633 from goat 1:300, Cy2 streptavidin 1:300 in BS
CCAP + neurobiotin	4% PFA in 0.1M PO_4 buffer	10% NGS, 0.25% BSA, 1% Triton X-100 in PBS	α-CCAP from rabbit (H Dircksen) 1:500 in BS	α-rabbit Alexa633 from goat 1:300, Cy2 streptavidin 1:300 in BS
cGMP + neurobiotin	4% PFA in 0.1M PO_4 buffer	5% NDS, 1% Triton X-100 in PBS	α-cGMP from sheep 1:1000 in BS	α-sheep Cy5 from donkey 1:300, Cy2 streptavidin 1:300 in BS
Dopamine + neurobiotin	0.1% GA and 10g/L SMB in 0.1M PO_4 buffer	5% NDS, 0.25% BSA, 1% Triton X-100, 8.5g/L SMB in PBS	α-dopamine from goat (HW Steinbusch) 1:1000 in BS	α-goat Cy5 from donkey (Jackson Immunoresearch) 1:300, Cy2 streptavidin 1:300 in BS
FMRFamide + neurobiotin	4% PFA in 0.1M PO_4 buffer	10% NGS, 0.25% BSA, 1% Triton X-100 in PBS	α-FMRFamide from rabbit (E Marder) 1:1000 in BS	α-rabbit Alexa633 from goat 1:300, Cy2 streptavidin 1:300 in BS

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Table2 – continued from previous page

Antigens	**Fixative**	**Blocking solution (BS)**	**1st antibodies**	**2nd antibodies**
FMRFamide + neurobiotin + cGMP	4% PFA in 0.1M PO_4 buffer	5% NDS, 1% Triton X-100 in PBS	α-FMRFamide from rabbit 1:1000, α-cGMP from sheep in BS	α-rabbit Cy2 from donkey 1:300, α-sheep Alexa633 from donkey 1:300, Cy3 streptavidin (Jackson Immunoresearch) 1:300 in BS
Serotonin + neurobiotin	4% PFA in 0.1M PO_4 buffer	10% NGS, 0.25% BSA, 0.5% Triton X-100 in PBS	α-serotonin from rabbit (Immunostar) 1:1000 in BS	α-rabbit Alexa633 from goat 1:300, Cy2 streptavidin 1:300 in BS
Serotonin + NOS	4% PFA in 0.1M PO_4 buffer	10% NDS, 0.25% BSA, 5% Triton X-100 in PBS	α-serotonin from goat 1:1000, α-uNOS from rabbit 1:20 in BS	α-goat Cy5 from donkey 1:300, α-rabbit Cy2 from donkey 1:300 in BS

4.3 Results

4.3.1 Components of the NO/cGMP system in the corpora allata

All components of the NO/cGMP system could be found in the CA. Fixation insensitive NADPHdiaphorase activity (Fig. 14A), positive immunoreactivity against the aminoacid citrulline (Fig. 14B-C), and cells that show cGMP immunoreactivity in response to stimulation with NO (Fig. 14D) were detected in the CA and CC.

The enzyme NOS is a diaphorase that can reduce NADPH (Davies, 2000). This activity is resistant to methanol/formalin fixation and can be used in a precipitation assay (Ott and Elphick, 2003). NADPH diaphorase activity serves as an indirect indicator for the presence of NOS. Strong NADPHd activity was observed in the CA (Fig. 14A). In addition, NOS immunoreactivity was also detected by an antibody staining against uNOS (see below Fig. 26A). Citrulline immunoreactive structures in the CA demonstrate active

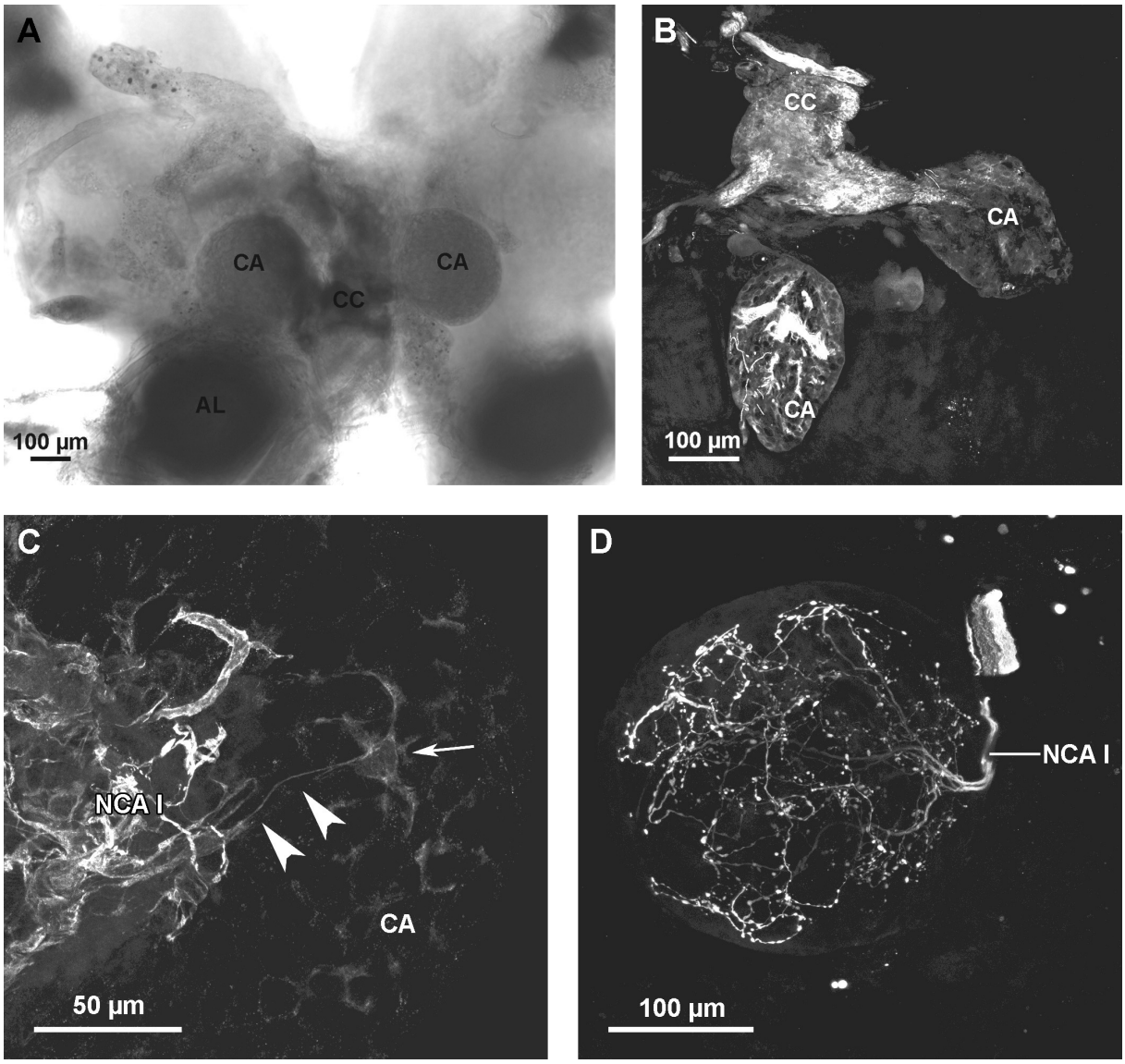


Figure 14: *NADPHdiaphorase activity, citrulline production, and cGMP accumulation in the CA*

A: Wholemount after NADPHd histochemistry. Strong NADPHd activity, indicated by the dark precipitate of nitroblue tetrazolium, can be found in the antennal lobes (AL), the corpora cardiaca (CC) and the corpora allata (CA). **B:** 50 μm thick section through the retrocerebral complex after immunostaining against citrulline. As a byproduct of NO synthesis, immunoreactivity against citrulline indicates NO production immediately before the dissection and fixation of the tissue. **C:** Section through a corpus allatum after immunostaining against citrulline. A cell in the CA parenchym (thin arrow) sends a process (arrowheads) toward the entry side of the nervus corporis allati I (NCAI). **D:** Section through a corpus allatum after immunostaining against cGMP. Fibers showed cGMP immunoreactivity after stimulation with the NO donor SNP. In contrast to the staining against citrulline, no cGMP immunoreactive somata can be found but a dense network of fibers with varicosities entering the CA by the nervus corporis allati I (NCA I).

NO production in the CA immediately before tissue fixation. Citrulline immunoreactivity was present in fibers (Fig. 14B) and in parenchymal cells (Fig. 14C), some of which exhibiting processes reaching into the NCA I.

Aminoguanidine hemisulfate (AG) inhibits NOS (Misko et al., 1993; Jianmongkol et al., 2000) and has been demonstrated to suppress NOS mediated accumulation of citrulline immunoreactivity in central brains of *Ch. biguttulus* (Weinrich et al., 2008). To test, whether citrulline immunoreactivity in the CA is sensitive to systemic application of an NOS inhibitor, animals were injected with either saline or AG dissolved in saline before the dissection of brain and retrocerebral complex. The intensity of citrulline immunoreactivity in the CA was considerably reduced in the AG treated group, compared to saline injected controls (Fig. 15).

While NO is produced by cellbodies and fibers in the CA, cGMP immunoreactivity was only detected in fibers branching in the CA (Fig. 14D). Varicosities in the cGMP immunopositive fibers indicated that these fibers may belong to peptidergic neurons (Santos et al., 2007).

To demonstrate that the antibody staining against cGMP detects the accumulation of cGMP as a specific reaction to the stimulation with NO, the cGMP accumulation protocol was conducted without adding the NO donor SNP and the results were compared with brains that received stimulation with NO (Fig. 16). In unstimulated brains, cGMP immunoreactivity was almost completely abolished. In unstimulated CA tissue, no varicose fibers immunopositive for cGMP as described above could be detected.

4.3.2 Neurons projecting from the brain to the corpora allata

Backfills of brain-to-CA connections were performed by cutting the right CA and placing it in a pipette filled with neurobiotin solution. This procedure labeled somata in the contralateral and ipsilateral PL and ipsilateral PI of *Ch. biguttulus* (Fig. 17A) as previously described in the literature for other orthopteran insects (Moore and Loher, 1988; Virant-Doberlet et al., 1994). In some preparations, also neurons in the contralateral PL were stained, (Fig. 17B). An exemplary overview is given in Fig. 17C.

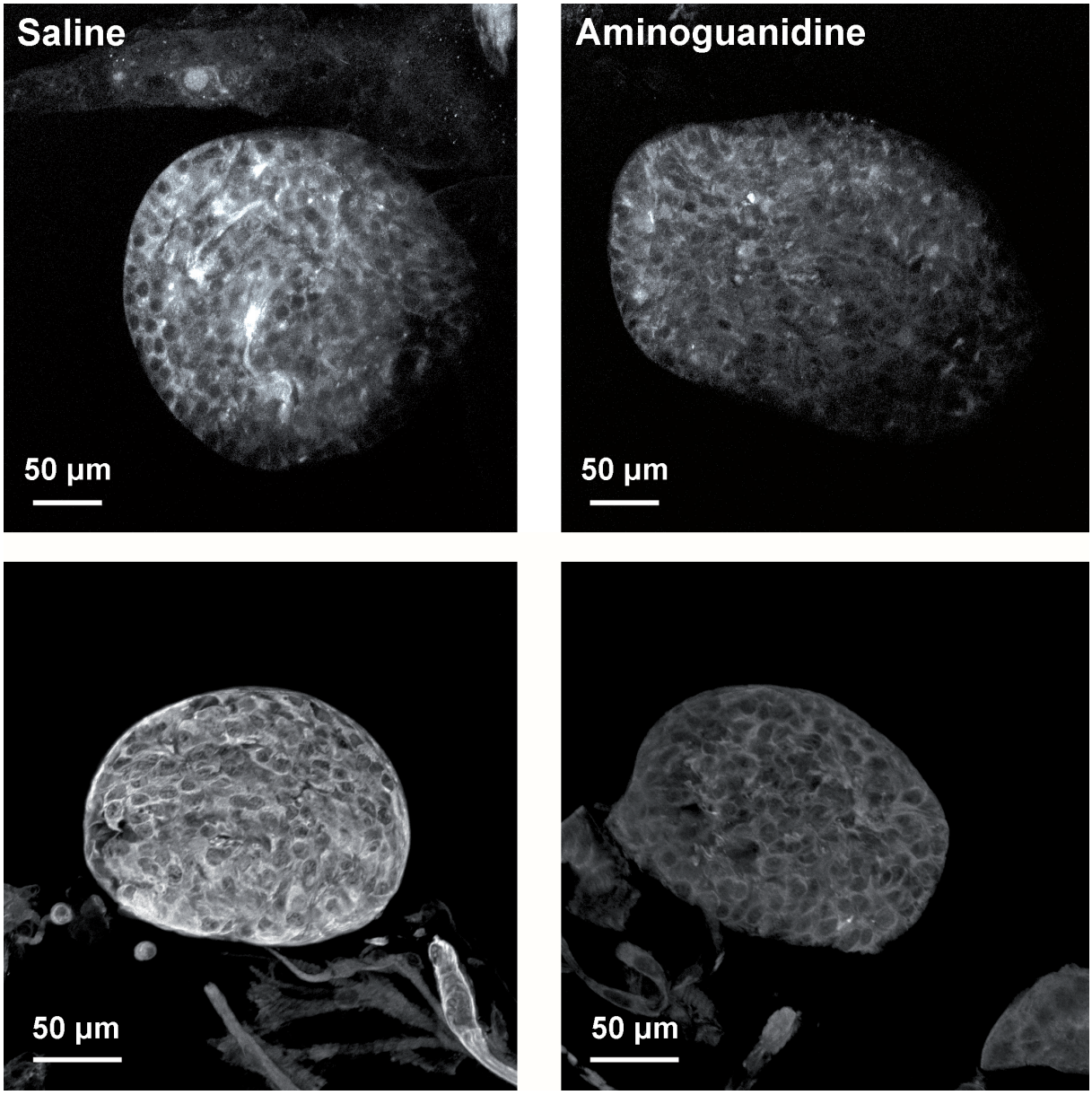


Figure 15: *Citrulline immunoreactivity in the CA after AG treatment*
Sections (50 μm) through the CA of female *Ch. biguttulus* after inhibition of NO production by aminoguanidine (maximal pixel values extracted). Four hours prior to tissue fixation, females were injected with 30 μL 10^{-2} M aminoguanidne dissolved in Locusta saline (Aminoguanidine) or with 30 μL Locusta saline (Saline). Citrulline immunoreactivity in the CA was not completely abolished in the CA tissue of the treated group but clearly reduced. The upper row shows a section through the center of the CA where most of the citrulline immunoreactive fibers arborize. The lower row shows more lateral sections through the parenchymal cells of the CA.

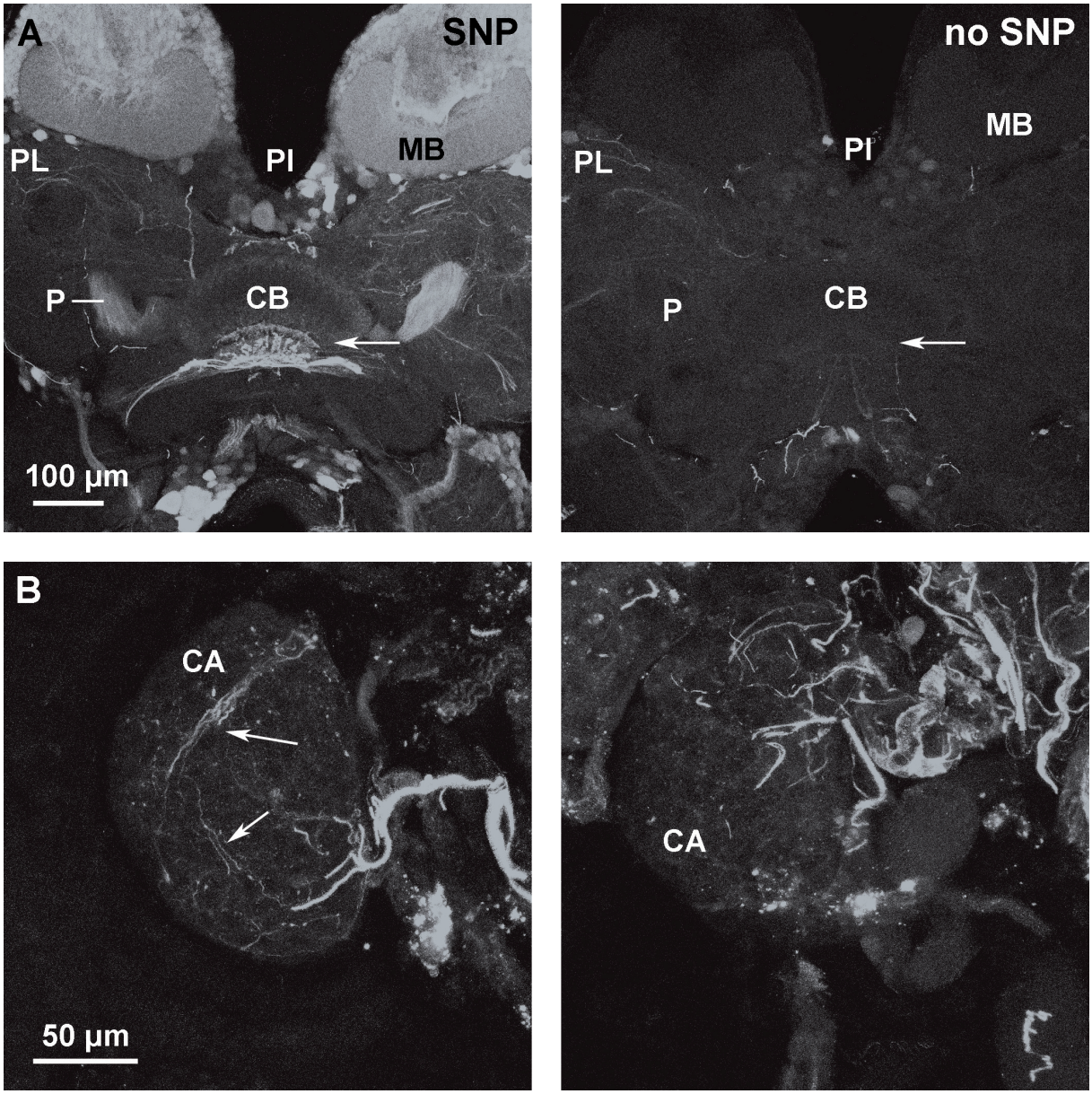


Figure 16: *Immunostaining of accumulated cGMP with and without stimulation by SNP* **A:** Section (27 μm) through the brains of two female *Ch. biguttulus* after immunostaining of cGMP, maximal pixel values extracted. In the left brain, cGMP accumulation was stimulated with the NO donor SNP. Strong immunoreactivity was found in the lower division of the central body (CB), pointed out by an arrow, in the pedunculi (P) of the mushroom bodies (MB) and in neurons of the pars intercerebralis (PI) and lateralis (PL). The right brain did not receive treatment with SNP. Here, no staining was detected in mushroom bodies, pedunculi and central body and only faint immunoreactivity occurred in cells in the PI and PL. **B:** Section (48 μm) through the corpora allata (CA) of two female *Ch. biguttulus* after immunostaining of cGMP, maximal pixel values extracted. In the SNP stimulated CA tissue (on the left) the typical varicose fibers can be found (arrows) while they are missing in the unstimulated CA, on the right. Bright areas in both images are artefacts derived from the staining of tracheal tissue.

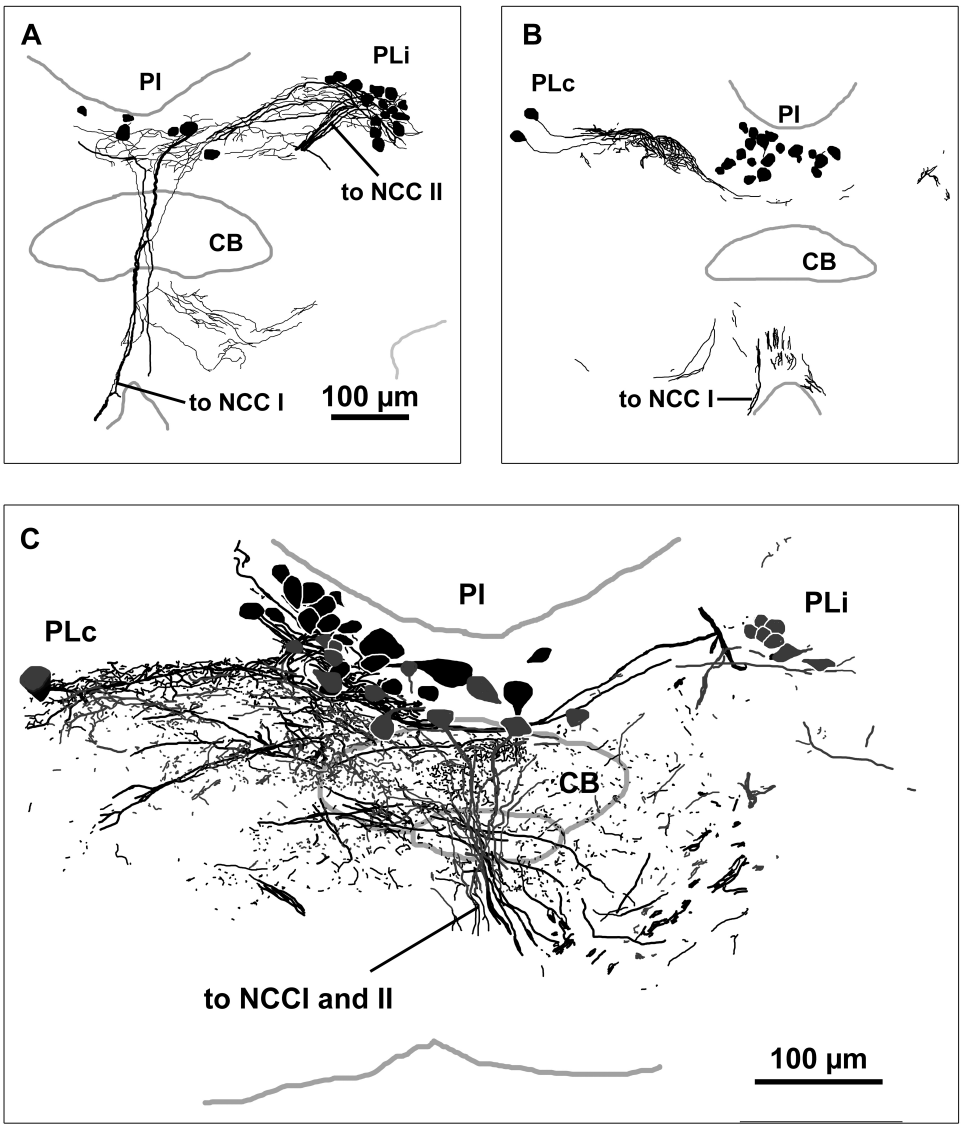


Figure 17: *Diagrams of brain neurons with connections to the right CA*
Drawing of neurobiotin traced neurons in the brain after backfill of the right CA. Drawings derived from horizontal brain sections with a thickness of 50 μm. A, B, and C represent three different backfill experiments. **A:** Stained somata are found in the pars intercerebralis (PI) and in the ipsilateral pars lateralis (PLi). A group of neurons in the PLi sends processes into the ipsilateral NCC II. Prominent fibers were also found in the region of the contralateral NCC I. **B:** In another experiment, two somata were also found in the contralateral pars lateralis (PLc). This diagram also shows a large group of neurons in the PI that contact the CA. CB = central body. **C:** With many somata stained in this backfill experiment, the scheme can serve as an overview of brain neurons contacting the right corpus allatum. Somata and fibers located dorsally from the central complex are illustrated in gray. A group of cells in the ipsilateral pars lateralis (PLi) can be found as well as a soma in the contralateral pars lateralis (PLc). In the pars intercerebralis (PI), a group of up to 30 neurons can be stained by backfill of the right CA. Their fibers, running dorsally of the central complex, reach the CA probably by both, the nervus corporis cardiaci I (NCC I) and II. CB = central body

4.3.3 Tracing NO producing fibers from the corpora allata to the brain

To identify the origin of NO producing fibers in the CA, the backfill technique was combined with immunocytochemistry against uNOS. Again, backfilled somata were found at the same sites as described before, but a colocalization between neurobiotin taken up at the CA and uNOS immunoreactivity was not detected. Although, uNOS immunopositive fibers are present in the median bundle which is known to contain axons running into the retrocerebral complex it was not colocalized with neurobiotin at this site (Fig. 18A).

In the PI and PL, where the somata of brain derived projections to the CA are localized, colocalization of uNOS immunoreactivity and neurobiotin was also not detected (Fig. 18B). Since stainings against uNOS often appear rather diffuse and with a high unspecific background (Weinrich et al., 2008), backfills of the CA were repeated and combined with immunocytochemical staining against citrulline. In accordance with uNOS immunocytochemistry, no citrulline immunoreactive neurons that also incorporated neurobiotin via backfills from the CA were labeled (Fig. 18C). These results suggest that NO production in the CA does not result from brain neuron derived innervations.

Various cell bodies in the CA displayed citrulline immunoreactivity and, hence, must have produced NO in recent periods preceding tissue fixation. Some of these cells appeared to send processes into the NCA I (Fig. 14C). It seemed likely that NO is produced by CA cells and might serve as a retrograde transmitter. This hypothesis was tested by backfills of the CC. By this technique, 5-10 cell bodies in the CA were labeled that must have taken up neurobiotin via processes in the CC (Fig. 19A). A subsequent immunostaining against citrulline demonstrated that CA cells which produce NO possess projections into the CC (Fig. 19B and C).

A first attempt to identify the brain regions that are innervated by those CA cells was made by introducing an incision to the brain at the height of the PL (Fig. 20A) and placing a dextrane-neurobiotin crystal into the opening. This experiment aimed to find out, whether CA cells project to this area.

One cell in the CA accumulated the dextrane via its cut processes in the brain. A subsequent immunostaining revealed that this cell was immunoreactive for uNOS antiserum

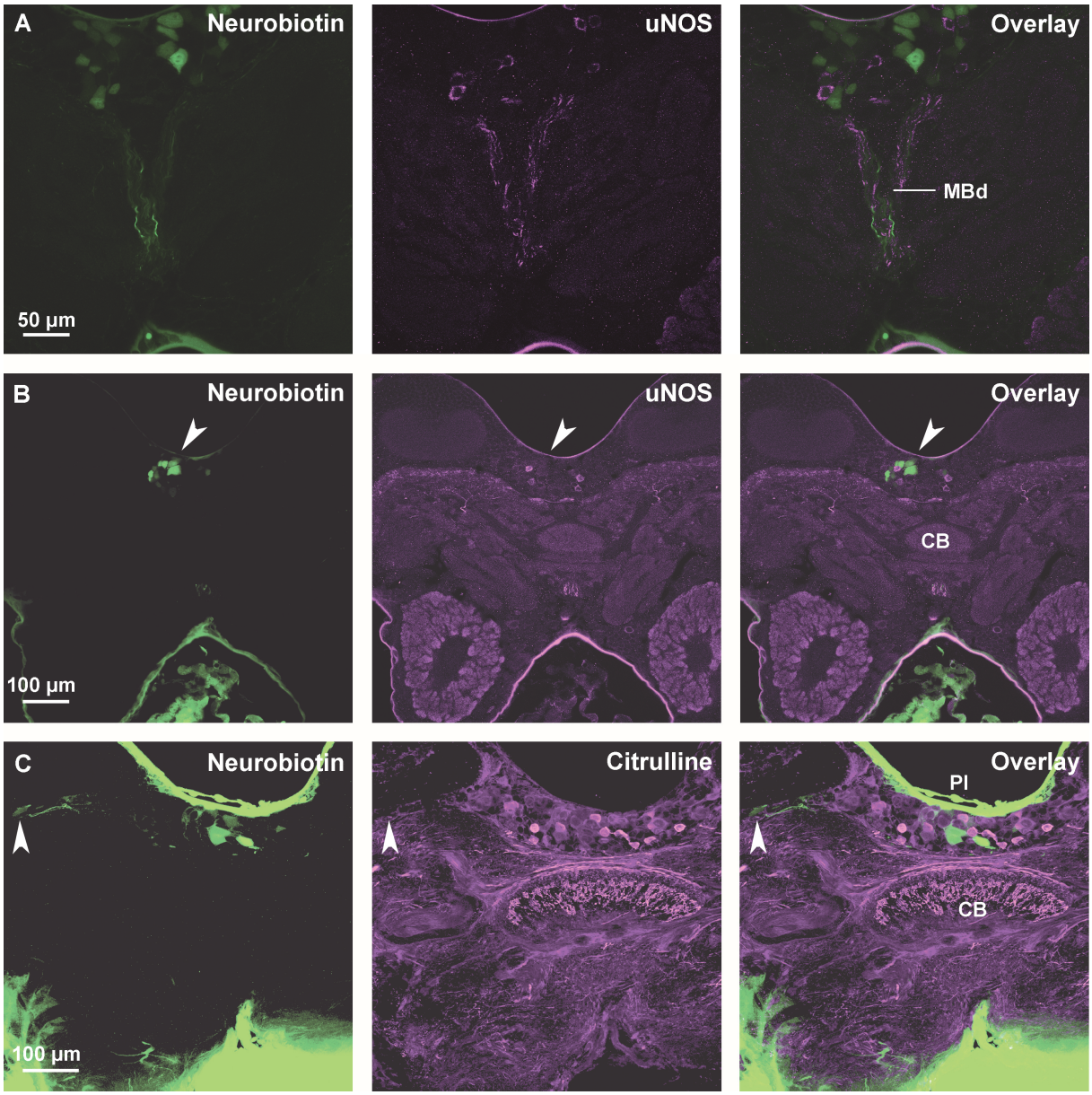


Figure 18: *Backfill of CA-to-brain connections combined with uNOS and citrulline immunocytochemistry*
Horizontal section (50 μm) after backfill at the right CA. Cells that incorporated the tracer are stained in green. Cells that are immunopositive for uNOS or citruline, respectively, are stained in purple. **A:** After backfill from the right CA, fibers positive for both, neurobiotin and uNOS immunoreactivity, were found in the median bundle (Mbd) but a colocalization of uNOS immunoreactivity and neurobiotin was not detected. **B:** A group of cells in the contralateral pars intercerebralis (arrow head) has direct connections to the right CA. None of the cells that took up the tracer showed positive immunostaining for uNOS. **C:** Backfill of brain-to-CA connections combined with citrulline immunocytochemistry. Neurons in the pars intercerebralis (PI) and the contralateral pars lateralis (arrowhead) took up the tracer. Staining against citrulline confirmed the results of anti-uNOS immunolabeling. Citrulline immunoreactivity is not present in brain neurons projecting to the CA. CB = central body.

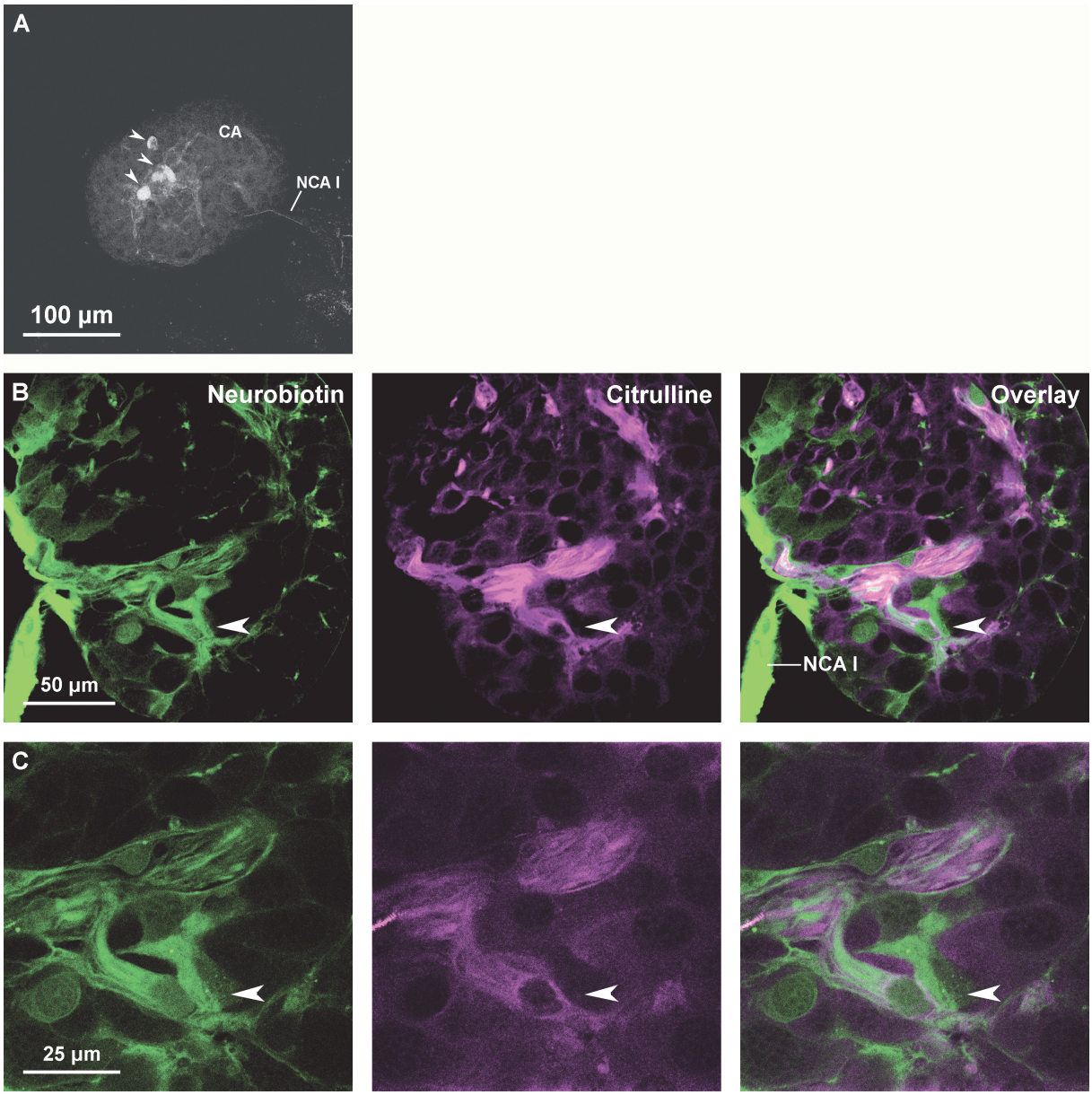


Figure 19: *Neurobiotin backfills of CA/CC connections and citrulline immunostaining in the CA*

A: Section of the right CA after neurobiotin tracing of CC/CA connections at the right CC. Arrowheads point to cells in the CA that have incorporated neurobiotin at the CC. **B and C:** Section of the right CA after neurobiotin tracing of CC/CA connections at the right CC and citrulline immunocytochemistry. **B:** Cells in the CA took up the neuronal tracer neurobiotin (green) from the CC via the nervus corporis allati I (NCA I). Some of these cell bodies were also immunopositive for citrulline (purple). Double labeling appears white in the overlay. The arrowhead points to a CA cell that projects to the CC and is citrulline immunoreactive. **C:** Magnification of a citrulline immunopositive cell with projections to the CC.

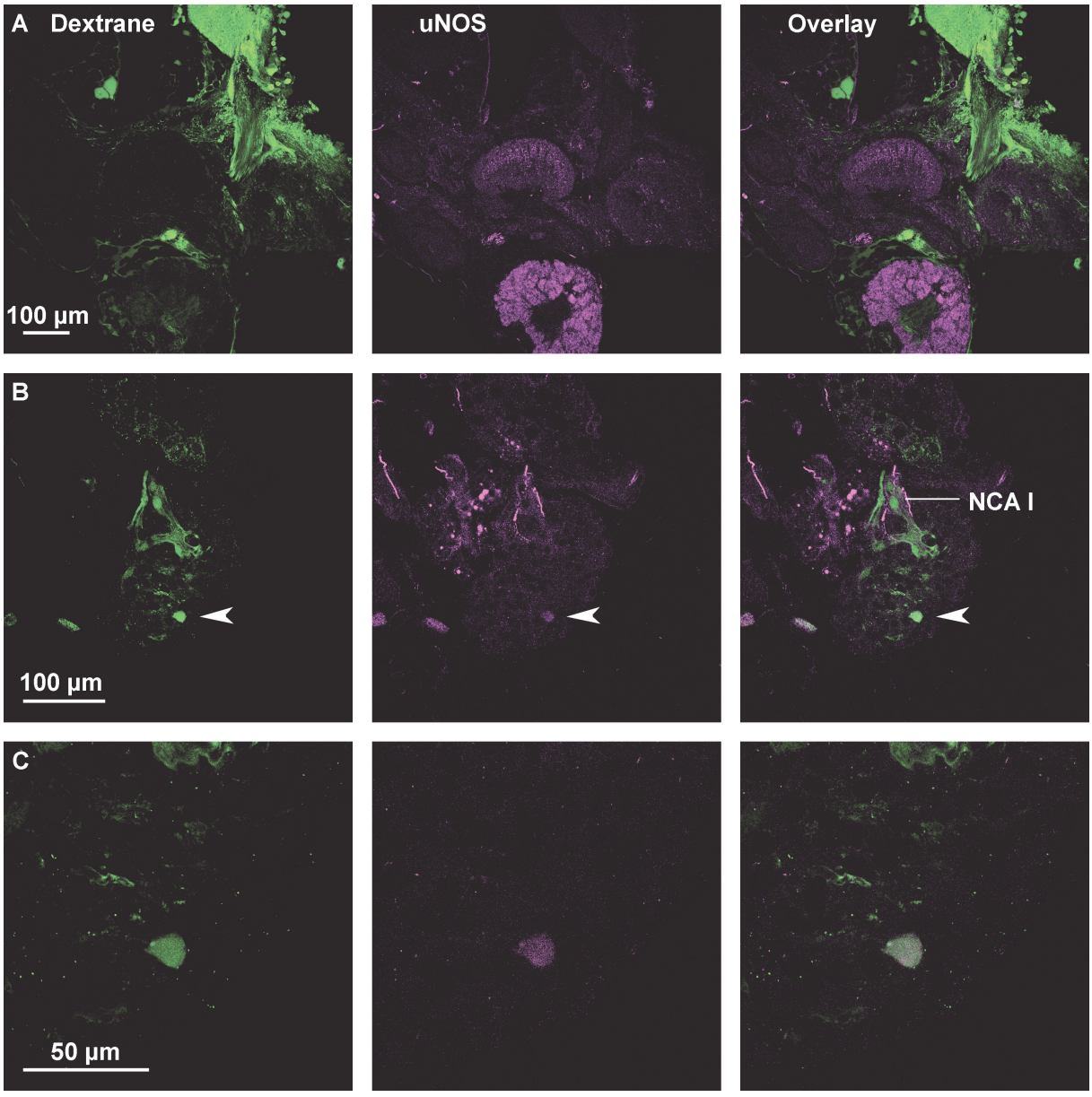


Figure 20: *Tracing the process of a CA cell into the brain*
The brain was incised below the right mushroom body at the height of the pars lateralis. A dextrane crystal was introduced into the cut and immunocytochemistry against uNOS was applied. **A:** Horizontal section through the brain shows the site of the incision. Dextrane labeling is green, uNOS immunoreactivity is purple. **B:** Section through the right CA. Fibers in the NCA I are stained and also a cell within the CA (arrowhead) that has taken up dextrane from the incision in the brain. **C:** The magnification of the stained cell in the CA shows that it is uNOS immunopositive.

(Fig. 20).

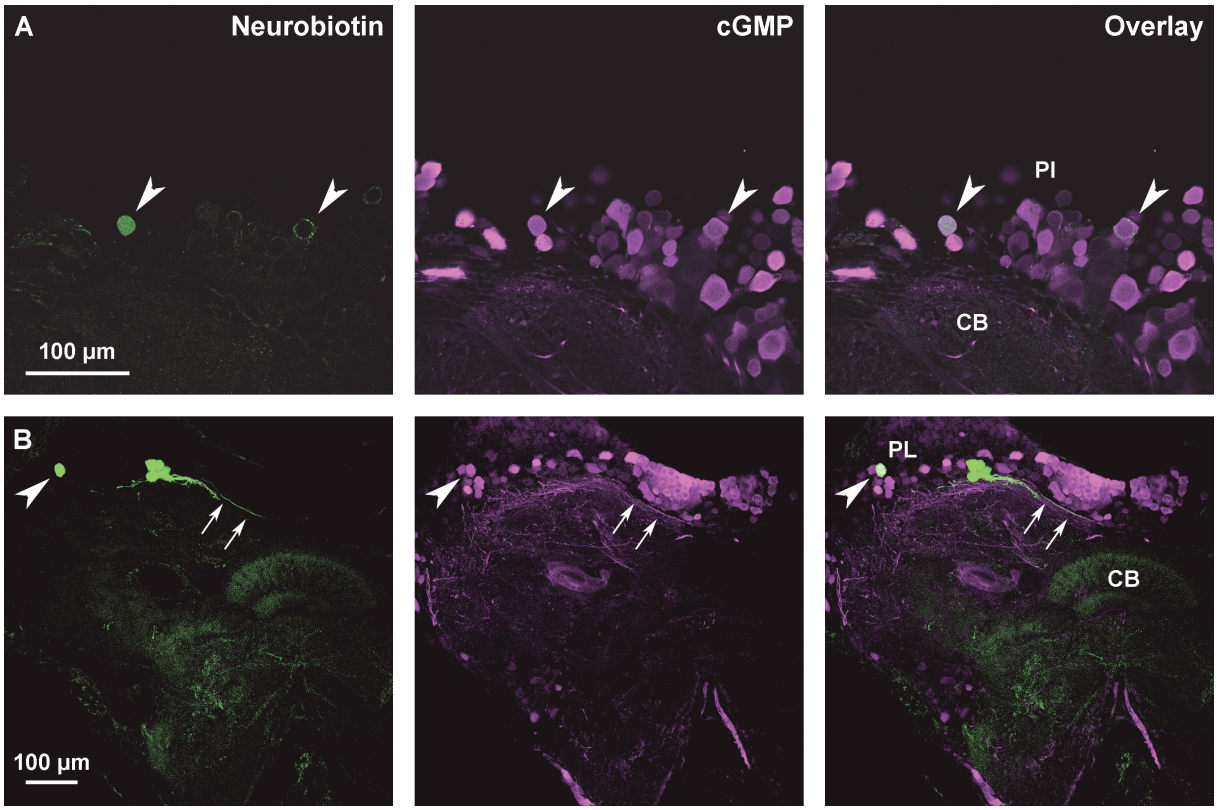


Figure 21: *Neurobiotin backfill of brain-to-CA connections combined with anti cGMP immunocytochemistry in the protocerebrum*
Horizontal brain sections (50 μm) after CA backfill with neurobiotin and NO stimulated cGMP accumulation. **A:** Many somata in the pars intercerebralis (PI) accumulated cGMP immunoreactivity in response to NO (purple) some of which have projections to the CA, stained in green. **B:** A cell in the contralateral pars lateralis (arrowhead) is positive for both, neurobiotin and cGMP immunoreactivity. Thin arrows point to a fiber probably crossing the brain to the ipsilateral side. PL = pars lateralis, CB = central body.

4.3.4 Tracing the origin of cGMP positive fibers in the corpora allata

Anti cGMP immunostainings revealed that only fibers but no cell bodies in the CA accumulate cGMP upon stimulation with NO (see Fig. 14D). These fibers innervate the CA by the NCA I and therefore must have originated either in the brain or in the CC. By combination of the CA backfill technique with cGMP immunocytochemistry following stimulation with NO, brain neurons responding to NO and projecting into the CA could be traced.

Somata positive for both, neurobiotin and cGMP immunoreactivity, were found in the PI (Fig. 21A) and in the contralateral PL (Fig. 21B). Fig. 22 shows a reconstruction of

neurons in the contralateral pars intercerebralis and the position of cGMP immunopositive neurons with projections to the CA in the PI. By its characteristic branching pattern this type of neuron in the distal part of the PL can easily be recognized in other backfills, too, for example in Fig. 17C.

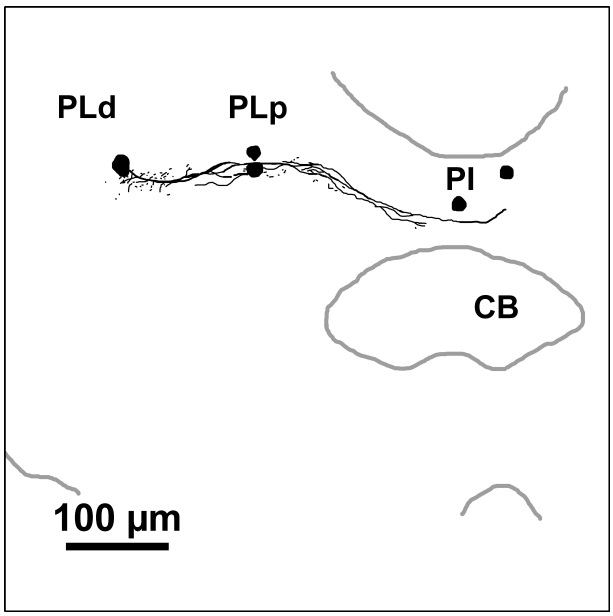


Figure 22: *Diagram of cGMP immunopositive neurons with connections to the contralateral CA*
By backfills of the right corpus allatum in combination with NO stimulation, cells in the contralateral pars lateralis with connections to the CA were identified that reacted to NO stimulation with the accumulation of cGMP and, hence, were cGMP immunopositive. A neuron in the distal pars lateralis (PLd) shows a prominent branching pattern that can be recognized in other backfill experiments, too. In addition, two neurons in the pars intercerebralis (PI) and two neurons in a more proximal part of the pars lateralis (PLp) were stained with both markers.

4.3.5 Allatostatin and -tropin in the corpora allata

A monoclonal antibody developed against *Diploptera punctata* allatostatin 7 was used to detect cells involved in the inhibitory control of JH production. While allatostatin immunopositive fibers were found in the whole retrocerebral complex (Fig. 23), cells projecting from the brain to the CA did not contain allatostatin immunoreactivity (Fig. 24A), indicating that the origin of allatostatin immunoreactivity in the CA does not result from brain neuron projections.

With an antibody raised against *Manduca sexta* allatotropin, no immunostaining

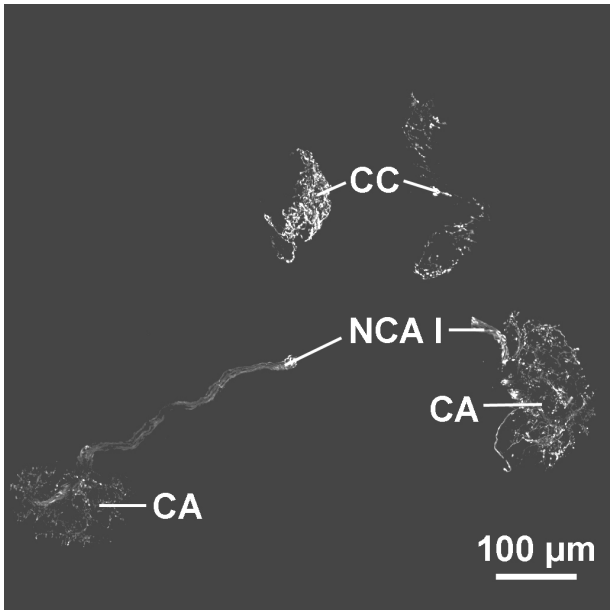


Figure 23: *Allatostatin immunoreactivity in the retrocerebral complex*
Anti Dip-allatostatin-7 immunoreactivity in the retrocerebral complex of a *Ch. biguttulus* female. Immunopositive fibers are present in the corpora cardiaca (CC), the corpora allata (CA), and the nervus corporis allati I (NCA I). No allatostatin immunopositive somata were found in the retrocerebral complex.

in the CA could be achieved. This result was supported by backfill experiments. Also here, no allatotropin immunopositive somata in the brain projecting to the CA could be detected (Fig. 24B). Though, a dense network of allatotropin immunopositive fibers was labeled in various neuropils of the central brain, including the central body. Mas-AT immunoreactive somata were found in PI and PL but none of these neurons innervated the CA.

In contrast to the experiments conducted with female *Ch. biguttulus*, similar stainings in *D. punctata* labeled somata in the brain that projected to the CA and contained allatostatin immunoreactivity (Fig. 25). In the PI (Fig. 25A-B) and PL (Fig. 25C-D), neurons were positive for both, Dip-Ast-7 immunoreactivity and the tracer neurobiotin. In addition, the distribution of Dip-Ast-7 immunopositive cells in *D. punctata* brains appeared to be different than in *Ch. biguttulus*. Though, not all Dip-Ast-7 immunopositive somata could be labeled by the CA backfill, they occurred in the same plane as those that took up neurobiotin from the CA backfill. In *Ch. biguttulus*, Dip-Ast-7 immunoreactive cells did not appear in the same layer as cells that project to the CA.

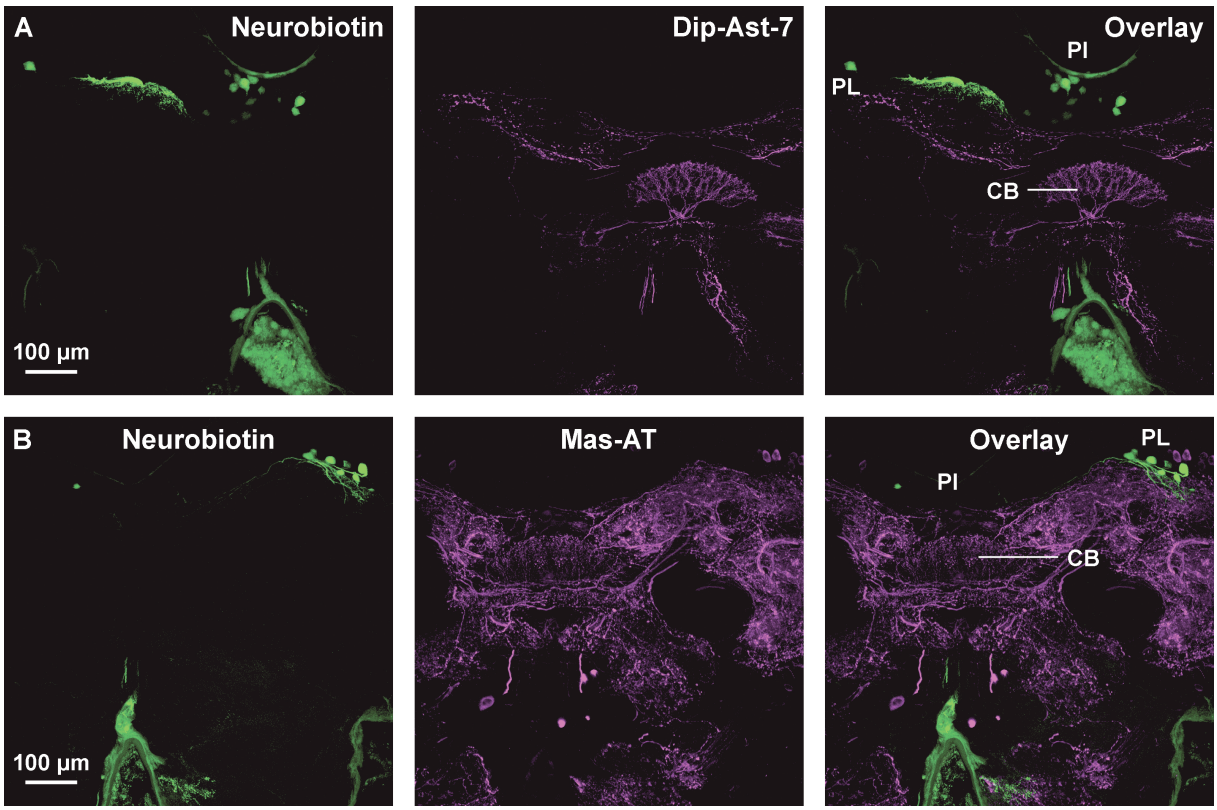


Figure 24: *Backfill of brain-to-CA projection neurons combined with allatostatin and -tropin immunocytochemistry*
A: Horizontal section through the brain (50 μm, maximal pixel values extracted) after backfill of the right corpus allatum with neurobiotin (green). Dip-allatostatin-7 immunoreactivity is displayed in purple. Though, allatostatin immunopositive neurons were present in the pars intercerebralis (PI) and lateralis (PL), they did not lie in the same plane as neurons projecting from the brain to the CA. **B:** Horizontal section through the brain (50 μm, maximal pixel values extracted) after backfill of the right corpus allatum with neurobiotin (green). Mas-AT immunoreactivity is marked in purple. In the PL, neurons immunopositive for allatotropin were located in the same plane as neurons projecting to the CA but a colocalization of neurobiotin and allatotropin immunoreactivity was not detected.

4.3.6 Relationship between the NO/cGMP system and juvenile hormone production control system

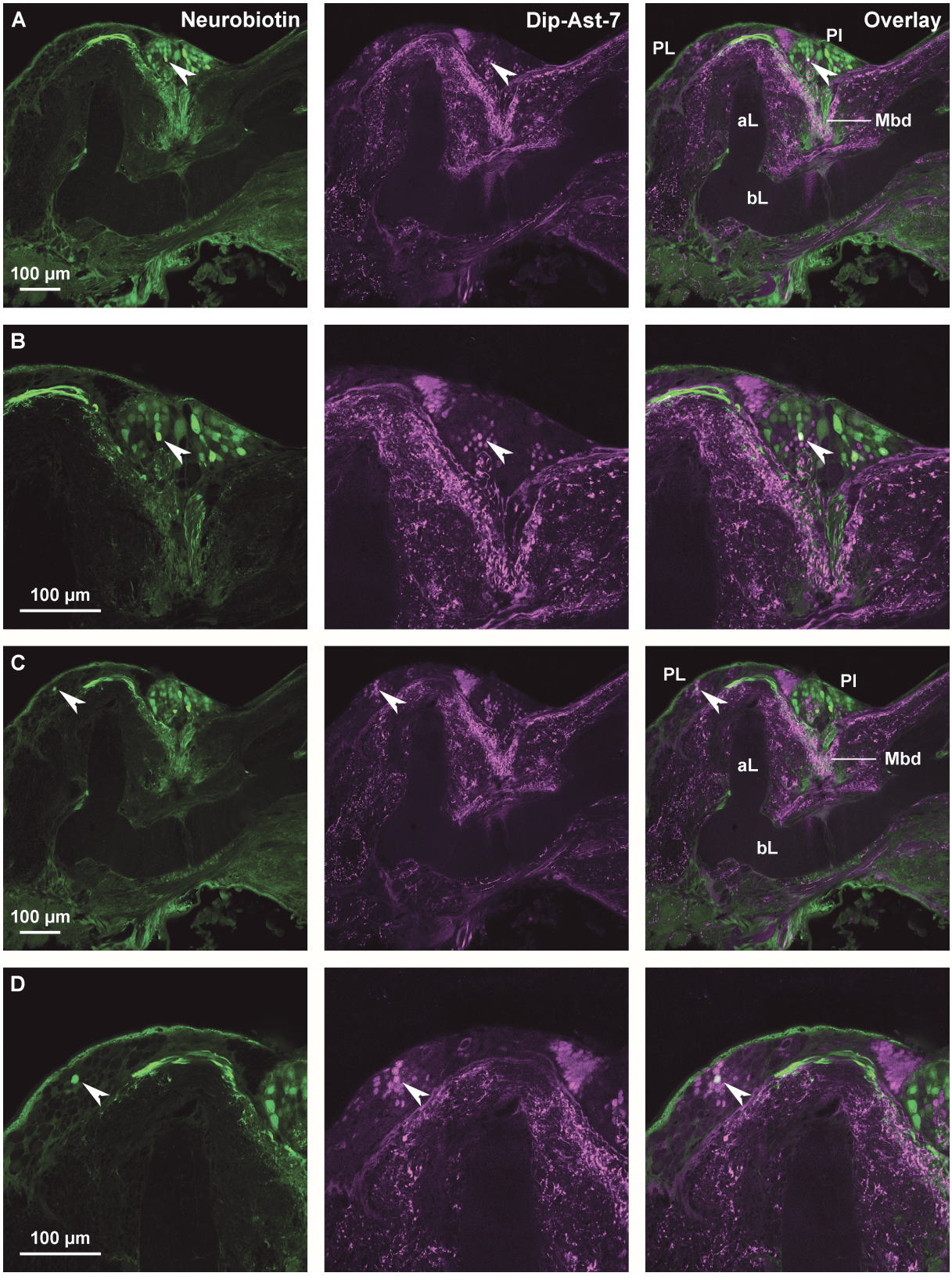
Both, Dip-Ast-7 immunoreactivity and Mas-AT immunoreactivity, were found in the same brain regions as neurons that produce NO (Kurylas et al., 2005). To evaluate if NO could be a co-transmitter of these peptides, double immunostainings against Dip-Ast-7 and uNOS were performed as well as immunostainings against Mas-AT and citrulline.

Allatostatin and uNOS immunoreactivity could both be detected in the CA but were not colocalized in the same fibers (Fig. 26A). The same was true for allatostatin immunopositive brain neurons. Allatostatin and uNOS immunoreactivity were both present in the upper division of the central body, but they occurred in different layers (Fig. 26B). As a third side of interest, the region of the median bundle was examined. Fibers running into the retrocerebral complex pass this region before entering the NCC I and II. Also here, a colocalization between uNOS and allatostatin immunoreactivity could not be detected (Fig. 26C).

Allatotropin immunoreactivity was detected with an antibody against *Manduca sexta*-allatotropin (Mas-AT) from rabbit. To find colocalizations between NO production sites and allatotropin, only the antibody against citrulline from mouse could be used because the uNOS antibody derived from the same species as the Mas-AT antibody. As described above, allatotropin immunoreactivity was not detectable in the CA. For this reason, only the brain was examined. However, a colocalization between citrulline and allatotropin immunoreactivity was not detected (Fig. 27).

Allatostatin and -tropin immunoreactive neurons do not produce NO but at least

Figure 25: *Backfill of brain-to-CA projection neurons combined with allatostatin immunocytochemistry in* Diploptera punctata
Horizontal section (50 μm) through the brain of a female *Diploptera punctata* after backfill of the right corpus allatum with neurobiotin (green) and immunostaining against Dip-allatostatin-7 (purple). **A:** In the pars intercerebralis (PI), a neurobiotin traced neuron was positive for allatostatin (arrowhead). **B:** magnification of the allatostatin positive neuron (arrowhead) in the PI region that projects to the right corpus allatum. **C:** Also the pars lateralis (PL) contained a soma that was positive for both, neurobiotin and allatostatin (arrowhead). **D:** Magnification of the pars lateralis region, arrowhead points to a soma with connection to the right corpus allatum and immunolabeled for allatostatin. aL = α lobe, bL = β lobe, Mbd = median bundle.


A
Neurobiotin
Dip-Ast-7
Overlay
PL
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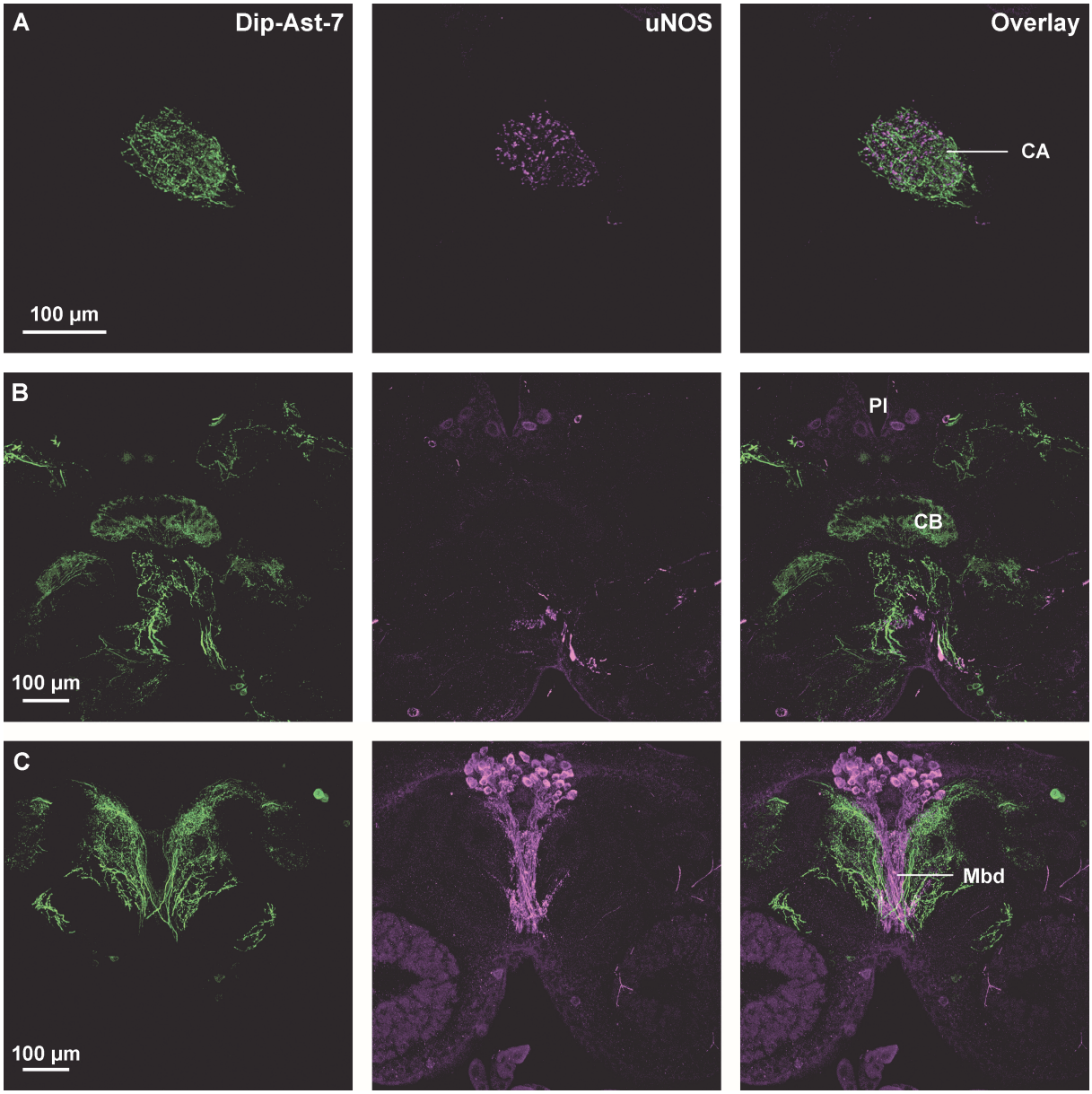

Figure 26: *Allatostatin and uNOS immunoreactivity in CA and brain*
A: Section (50 μm) through a corpus allatum (CA) after immunostaining against *Diploptera punctata* allatostatin 7 (green) and uNOS (purple), maximal pixel values extracted. Although, immunoreactivity against both substances can be found in the CA, there was no colocalization detected. **B and C:** Horizontal sections (50 μm) through the brain of a female *Ch. biguttulus* after immunostaining against allatostatin (green) and uNOS (purple). **B:** Allatostatin immunoreactivity can be found in the central body region (CB), but not in the same fibers as uNOS immunoreactivity. The uNOS immunopositive neurons in the pars intercerebralis (PI) do not contain allatostatin immunoreactivity. **C:** Fibers from the median bundle (Mbd) enter the NCC I and II which supply the CC and also the CA via the NCA I. Allatostatin and uNOS immunoreactivity were both detectable in median bundle fibers but were not colocalized.

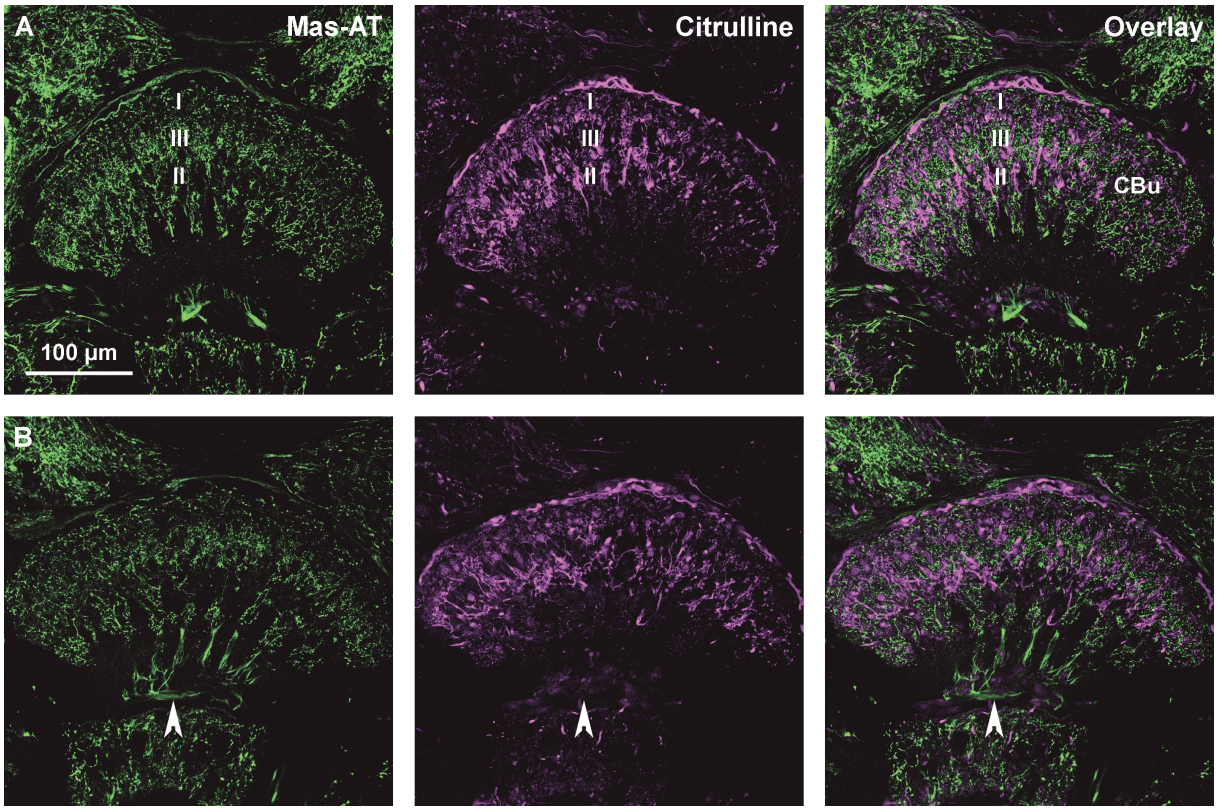

Figure 27: *Double immunostaining against Mas-AT and citrulline in the brain*
Horizontal sections (50 μm) through the brain of a female *Ch. biguttulus* after immunostaining against Mas-AT (green) and citrulline (purple). **A:** As described for *Schistocerca gregaria* (Homberg et al., 2004) Mas-AT immunoreactivity occurs in almost every layer of the central body's upper division (CBu) and is most prominent in layer III. Citrulline immunostaining is more dominant in layers I and II and the two substances do not occur in the same fibers. **B:** Similar to the distribution of Mas-AT immunoreactivity in *Schistocerca gregaria*, Mas-AT immunoreactivity is present in the fibers of MT1 tangential neurons (arrowhead) which have their somata in the pars intercerebralis and branch in the upper division of the central body. These fibers were free of citrulline immunoreactivity.

in the brain they seem to be targets of NO as double immunostainings against cGMP and the two peptides showed (Fig's. 28-31). cGMP is produced in response to NO by soluble guanylate cyclase. To accumulate cGMP in all NO-responsive cells, brains were stimulated with the NO donor SNP while phosphodiesterase activity was suppressed by Zaprinast.

In the pars intercerebralis (Fig. 28) and the inferior protocerebrum, some cells contained allatostatin immunoreactivity and were also immunopositive for cGMP. Fig. 29 was constructed after a double immunostaining experiment against allatostatin and cGMP to provide an overview of the locations of cells that were immunopositive for both substances.

Allatostatin immunopositive fibers in the CA did not react to NO, immunoreactivity against cGMP could not be detected in these fibers after stimulation with NO (Fig. 33A).

More than 80 cells, immunopositive for Mas-AT, that responded to NO with the ac-

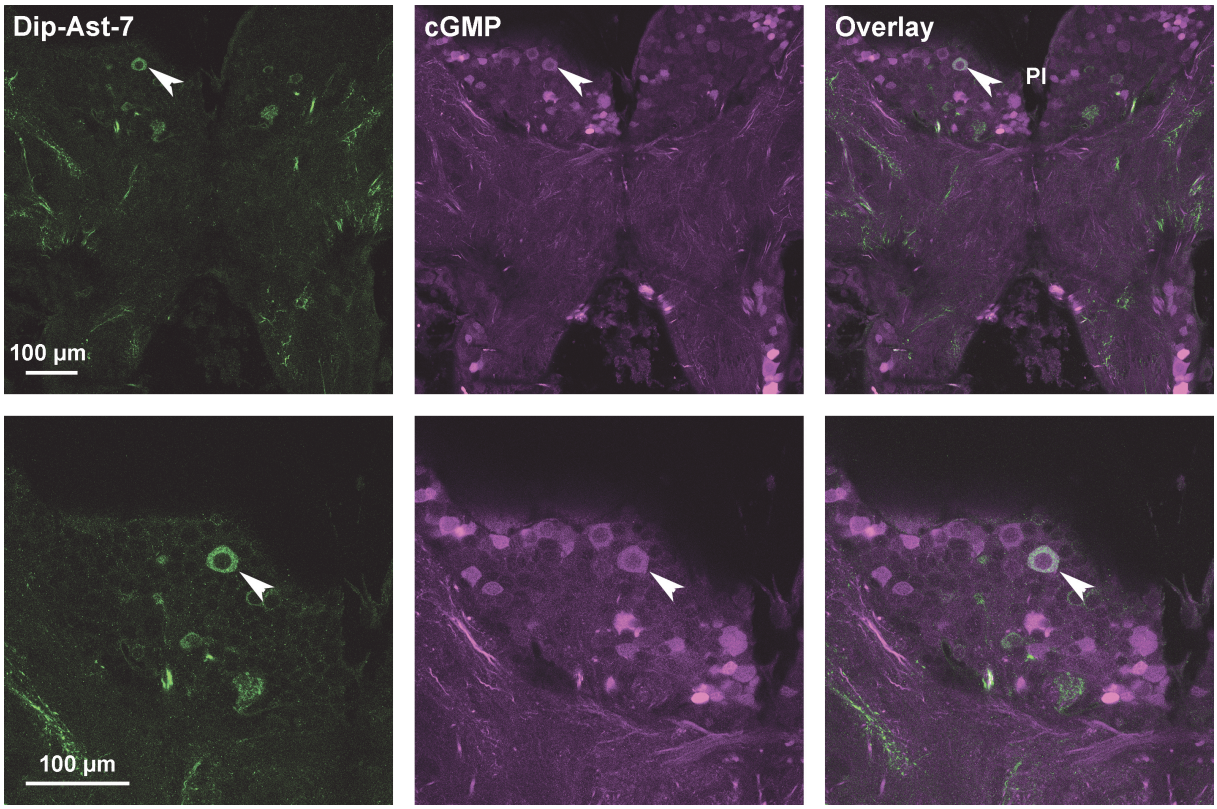

Figure 28: *Allatostatin and cGMP immunoreactivity in the pars intercerebralis*
Horizontal section (50 μm) through the brain of a female *Ch. biguttulus* after double immunostaining against Dip-Ast-7 (green) and cGMP (purple). The upper row shows the location of a neuron (arrowhead) in the pars intercerebralis (PI) that contained allatostatin and also cGMP immunoreactivity after NO stimulation. The lower row represents a magnification of the same neuron.

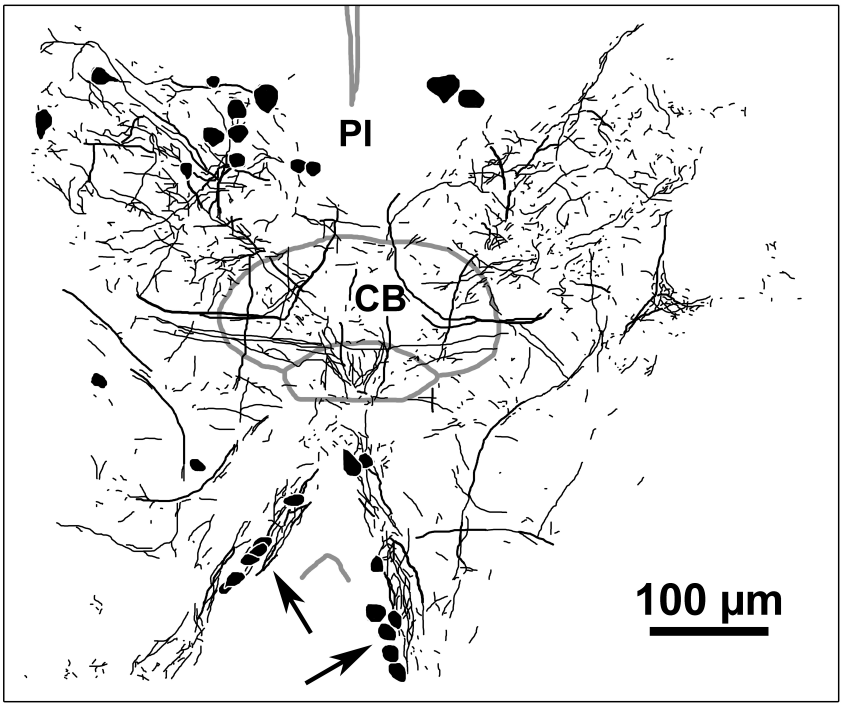

Figure 29: *Locations of NO-responsive allatostatin immunopositive neurons*
Reconstruction of somata and fiber locations from a horizontal section series through the brain of a female *Ch. biguttulus*. The tissue was stimulated to accumulate cGMP with the NO donor SNP. Afterwards, a double immunostaining against cGMP and Dip-Ast-7 was performed. The diagram shows cells and fibers that were positive for both substances. Somata were found in the pars intercerebralis (PI) and in the inferior protocerebrum (arrows). CB = central body.

cumulation of cGMP were found in the pars lateralis (Fig. 30). Also in other parts of the cortex such as the pars intercerebralis ($\sim$ 50) and the inferior protocerebrum ($\sim$ 40), allatotropin immunopositive neurons appeared to be targets of NO (Fig. 31).

4.3.7 Other transmitters of neurons innervating the corpora allata

Immunostaining against cGMP detected varicose fibers in the CA (Fig. 14D). Varicosities are frequent characteristics of peptidergic and/or aminergic neurons (Helle et al., 1995; Santos et al., 2007). For this reason, backfills of the CA were combined with immunostainings against crustacean cardioactive peptide (CCAP), FMRFamide, dopamine and serotonin.

FMRFamide immunoreactivity was detected in neurons of the PL and in somata in the PI. Somata in the PI had a connection to the right corpus allatum and were positive for neurobiotin (Fig. 32A). In the contralateral PL, one FMRFamide immunopositive cell

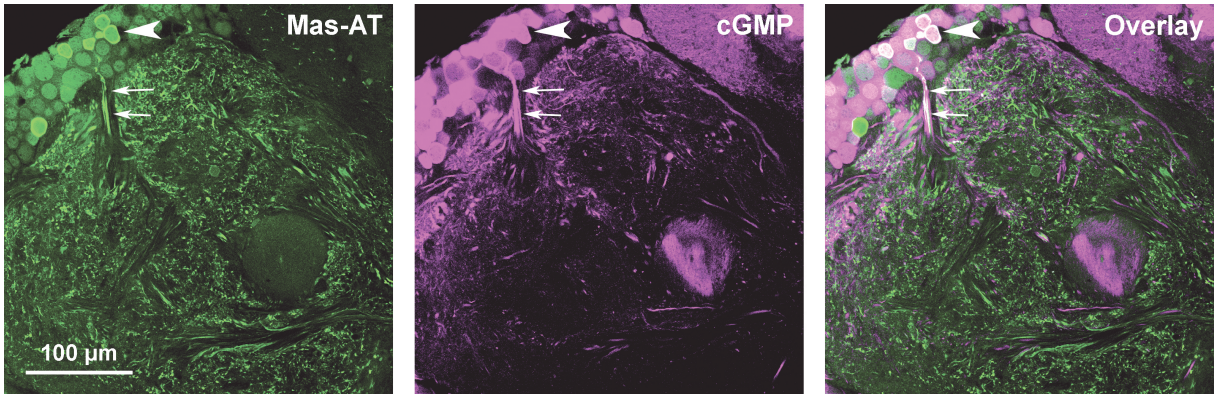

Figure 30: *Allatotropin and cGMP immunoreactivity in the pars lateralis*
Horizontal section (50 μm) through the brain of a female *Ch. biguttulus* after NO stimulated accumulation of cGMP and immunostaining against Mas-AT (green) and cGMP (purple). Colocalizations appear in white. Neurons in the pars lateralis (arrowhead) were immunopositive for both substances. They send fiber tracts to medial parts of the brain (arrows).

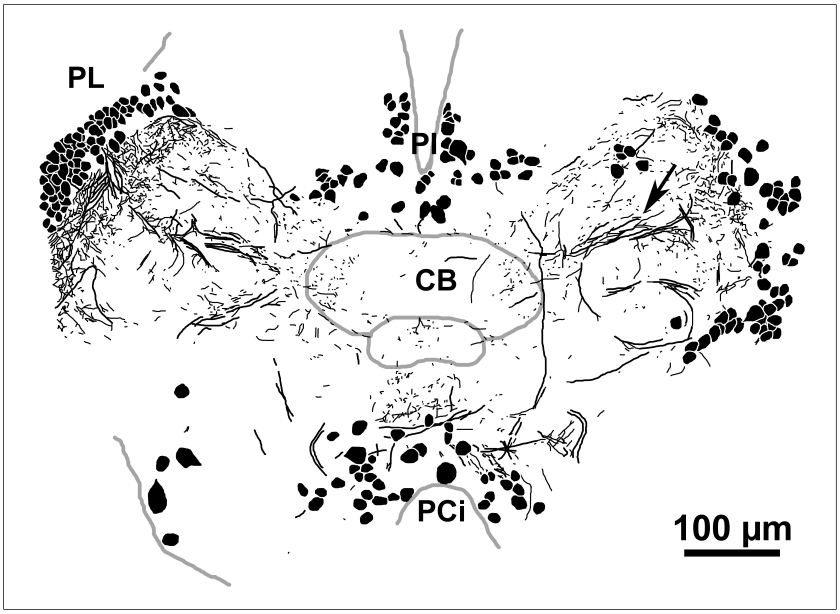

Figure 31: *Locations of NO-responsive allatotropin immunopositive neurons*
Reconstruction of somata and fiber locations from a horizontal section series through the brain of a female *Ch. biguttulus*. The tissue was stimulated to accumulate cGMP with the NO donor SNP. Afterwards, a double immunostaining against cGMP and Mas-AT was performed. The diagram shows cells and fibers that were immunopositive for both substances. Somata were found in the pars lateralis (PL) that send fiber tracts to medial parts of the brain (arrow). Many allatotropin neurons that are targets of NO are also located in the pars intercerebralis (PI) and the inferior protocerebrum (PCi). CB = central body.

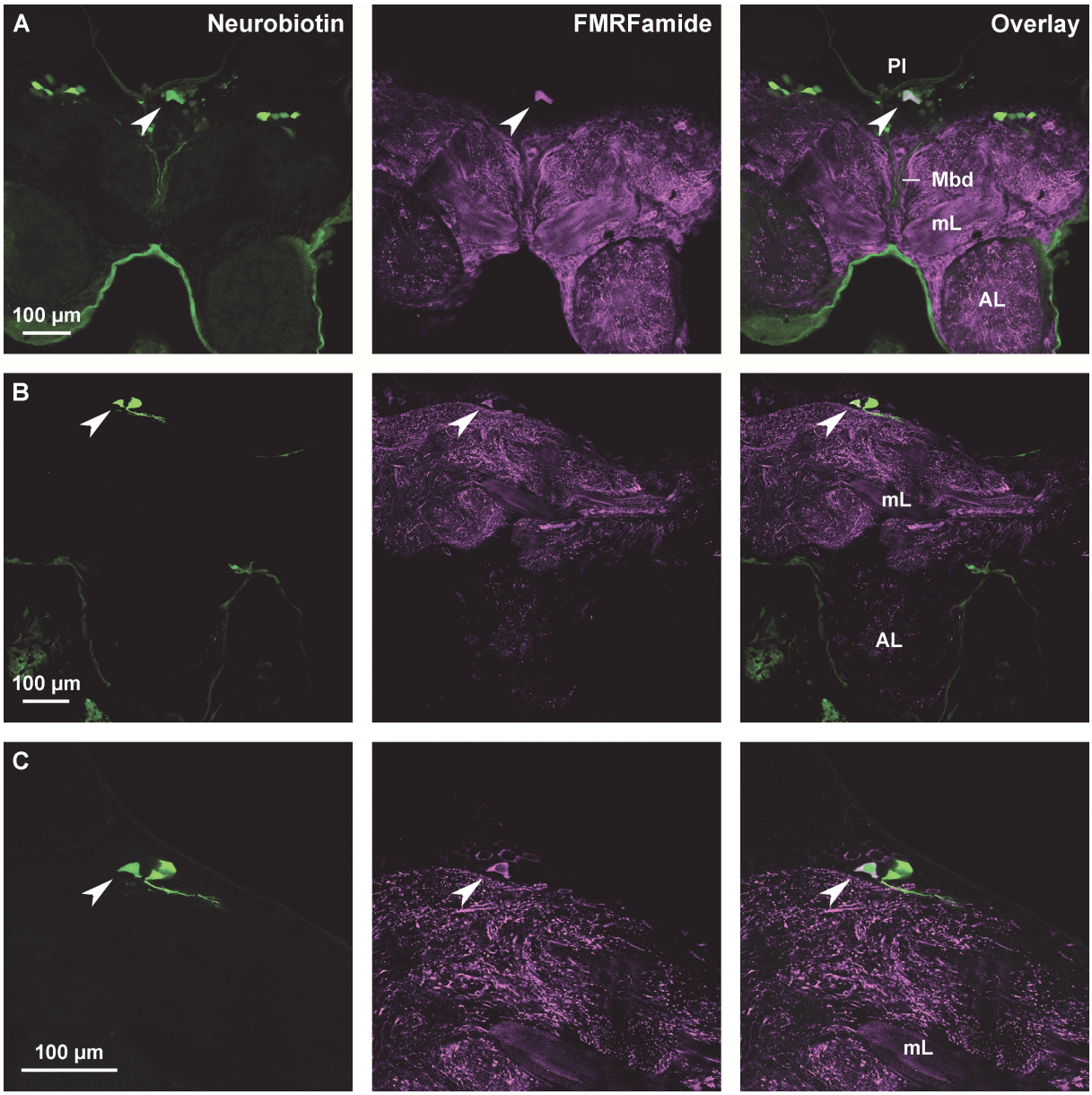

Figure 32: *Backfill of brain-to-CA projection neurons combined with FMRFamide immunocytochemistry*
Horizontal sections (50 μm) through the brain of a female *Ch. biguttulus* after backfill of the right corpus allatum with neurobiotin (green) and immunocytochemical staining against FMRFamide (purple). **A:** In the pars intercerebralis (PI), a soma with projections to the right corpus allatum was detected that was immunopositive for FMRFamide (arrowhead). Neurobiotin positive fibers can be found in the median bundle (Mbd) but those do not contain FMRFamide immunoreactivity. **B:** Also in the pars lateralis, FMRFamide immunopositive neurons can be found. The arrowhead points to a FMRFamide immunopositive soma with projection to the CA. **C:** Magnification of FMRFamide immunopositive cells in the pars lateralis. mL = medial lobe, AL = antennal lobe.

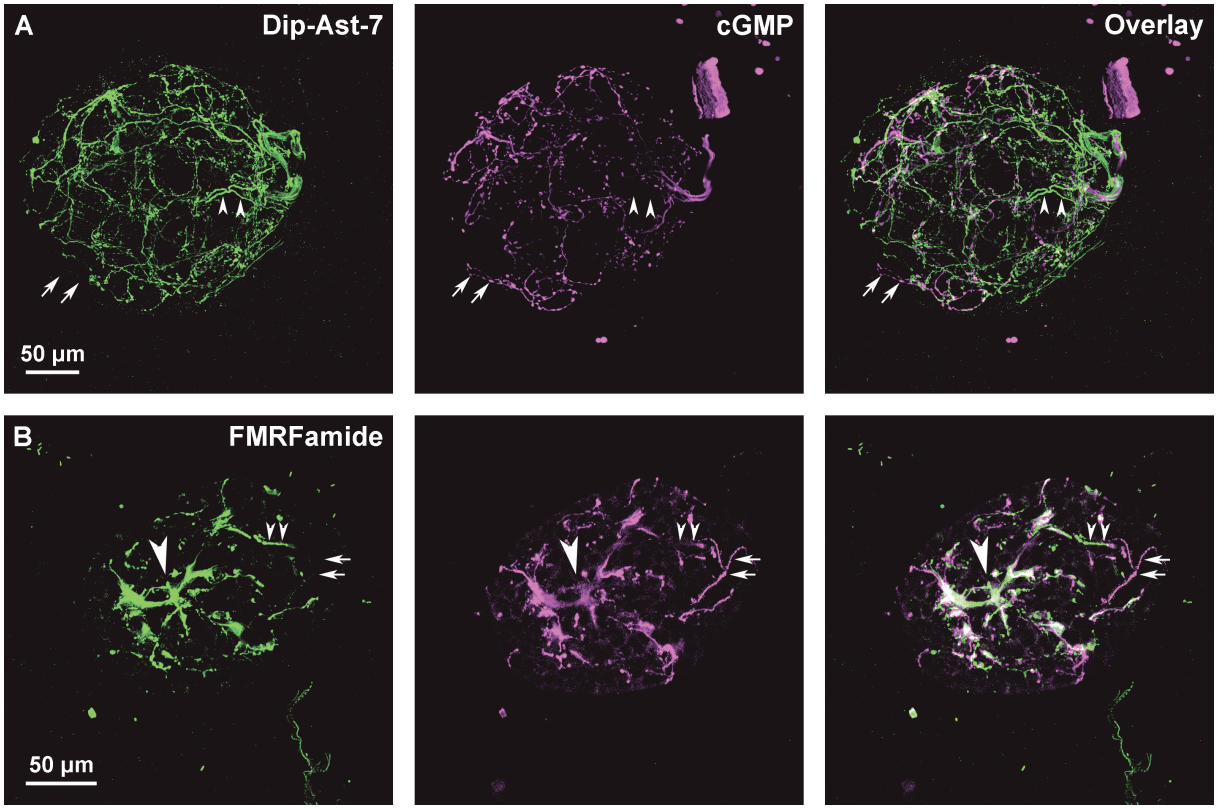

Figure 33: *cGMP, allatostatin, and FMRFamide immunoreactivity in the CA*
Horizontal sections (50 μm) through the corpus allatum of female *Ch. biguttulus* after NO stimulated cGMP accumulation, maximal pixel values extracted. **A:** cGMP immunostaining in combination with Dip-Ast-7 immunocytochemistry. cGMP immunopositive fibers (small arrows, purple) are different from allatostatin immunopositive fibers (small arrowheads, green). Apparent colocalizations (white color in overlay) are due to fiber crossings. **B:** cGMP immunostaining in combination with FMRFamide immunocytochemistry. In the majority of fibers, cGMP and FMRFamide immunoreactivity are colocalized (big arrowhead), while also cGMP only immunopositive fibers (small arrows) and FMRFamide only immunopositive fibers (small arrowheads) occur.

was detected that projected to the right CA (Fig. 32B).

In the CA, most fibers that reacted to NO with the accumulation of cGMP were immunopositive for FMRFamide, too (Fig. 33B). However, colocalization of both molecules was not 100%. Some FMRFamide immunopositive fibers showed no cGMP immunoreactivity and vice versa. This situation was reflected in the distribution of somata in the brain that were immunopositive for FMRFamide, accumulated cGMP, and/or had connections to the right CA. After backfill of the right CA with neurobiotin, cells were found that were positive for all three substances and others that were exclusively immunoreactive to cGMP or FMRFamide (Fig. 34).

The projections of FMRFamide immunoreactive fibers to the CA were running

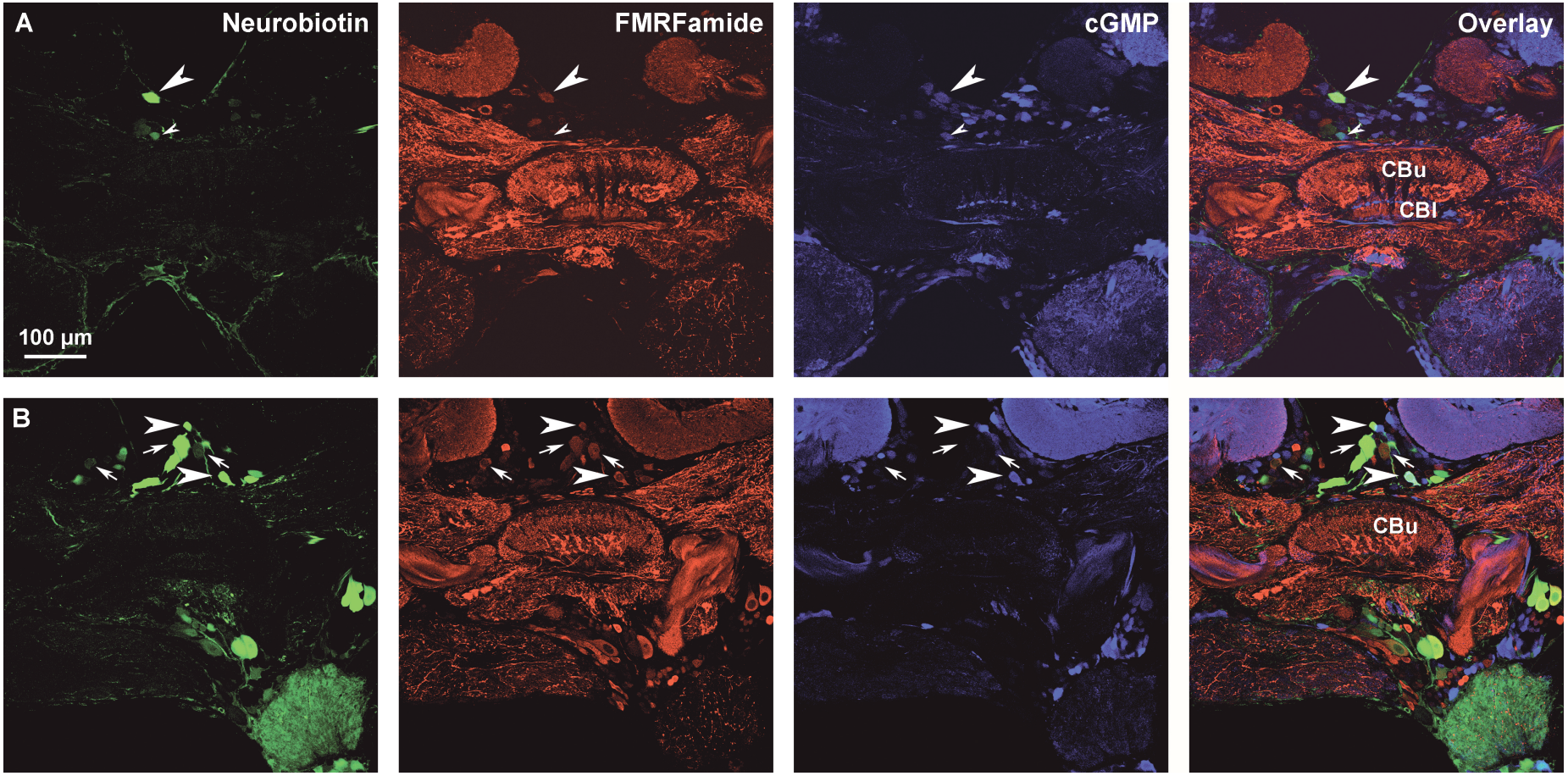

Figure 34: *Immunostaining of FMRFamide and cGMP after neurobiotin backfill of the right CA*

Horizontal sections (50 μm) through the brain of female *Ch. biguttulus* after backfill of the right CA with neurobiotin, NO stimulated cGMP accumulation and immunostaining against FMRFamide and cGMP. **A:** In this experiment, few neurons in the pars intercerebralis incorporated neurobiotin during the CA backfill. The large arrowhead points to a FMRFamide immunopositive neuron that has accumulated cGMP upon NO stimulation and projects to the right CA. Another neuron with connection to the CA (small arrowhead) is immunopositive for cGMP only. cGMP and FMRFamide immunoreactivity are not colocalized in the central body. While FMRFamide immunoreactivity was found in both, the upper and lower division (CBu and CBl), cGMP immunoreactivity occurred only in the lower division. **B:** In a different backfill experiment, a larger number of neurons in the PI was stained by the tracer. Besides neurons immunopositive for FMRFamide, cGMP, and neurobiotin (large arrowheads), also somata with connection to the CA were found that were only immunopositive for FMRFamide (small arrows).

through the median bundle, as backfills of brain-to-CA projections neurons showed (Fig's. 35 and 36). As described for *Manduca sexta* (Homberg et al., 1990), the peduncles of the mushroom bodies and the β lobes contained FMRFamide immunopositive fibers. Some of them were also immunopositive for cGMP and positive for neurobiotin (Fig. 36).

Immunoreactivity against the biogenic amine dopamine was present in the CA (Fig. 37A). The antibody against dopamine labeled cells and fibers. But, although dopamine immunoreactivity occurred in pars lateralis and pars intercerebralis of the brain, it was not detected in cells projecting to the CA (Fig. 37B and C).

Serotonin immunoreactivity occurred in the same layer as neurons stained by a CA backfill but neurobiotin and immunolabeling against this amine were not colocalized (Fig. 38)

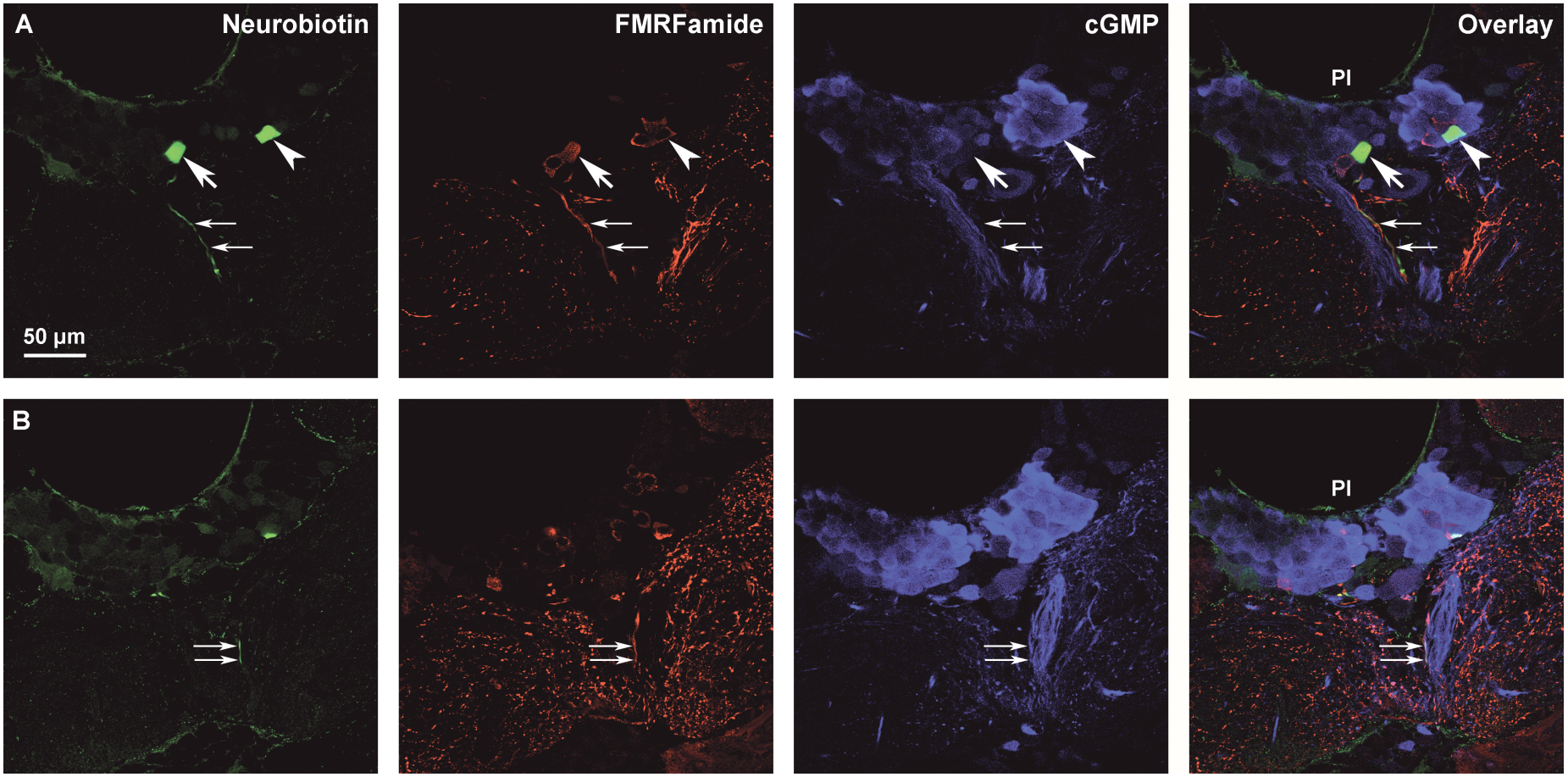

Figure 35: *cGMP and FMRFamide immunoreactive fibers in the median bundle*

Horizontal sections (50 μm) trough the brain of a female *Ch. biguttulus* at the level of the median bundle after backfill of the right CA with neurobiotin, NO stimulated cGMP accumulation, and immunostaining against FMRFamide and cGMP. **A:** At this level, the tracer stained two neurons in the pars intercerebralis (PI), one immunopositive for FMRFamide only (big arrow), one immunopositive for both, cGMP and FMRFamide (big arrowhead). The projection of the FMRFamdie immunopositive neuron can be followed in the median bundle (small arrows). **B:** At this level, the projection of a neuron immunopositive for FMRFamide and cGMP is seen in the median bundle (small arrows). PI: pars intercerebralis.

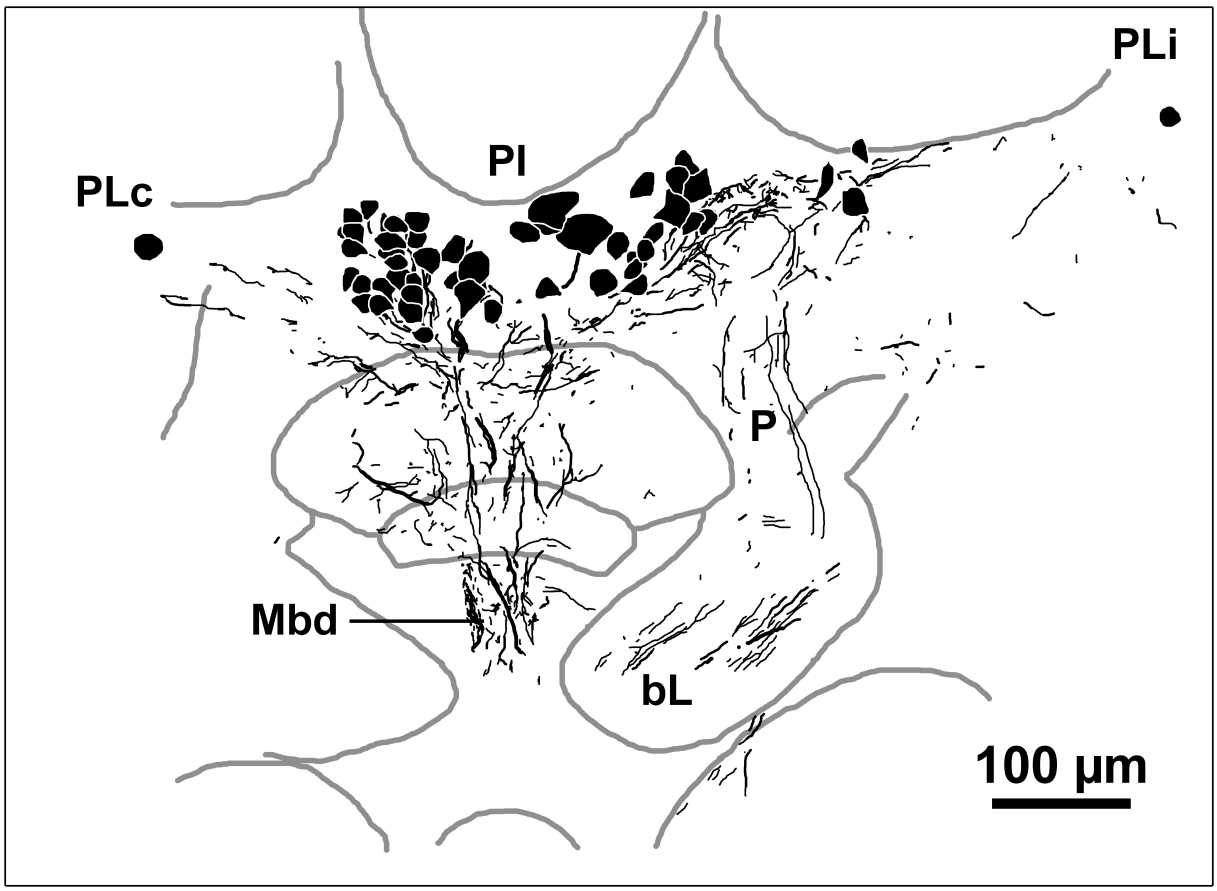

Figure 36: *Diagram of FMRFamide immunopositive neurons that respond to NO and project to the CA*

Reconstruction of somata and fiber locations from a horizontal section series through the brain of a female *Ch. biguttulus*. The right CA was backfilled with neurobiotin. The tissue was stimulated to accumulate cGMP with the NO donor SNP. Afterwards, a triple immunostaining against neurobiotin, cGMP, and FMRFamide was performed. The diagram shows cells and fibers that were positive for all three substances. Somata were found in the pars intercerebralis (PI) and in the contralateral and ipsilateral pars lateralis (PLc and PLi). Fibers were running through the median bundle (Mbd), the peduncle of the right mushroom body (P), and the β lobe (bL).

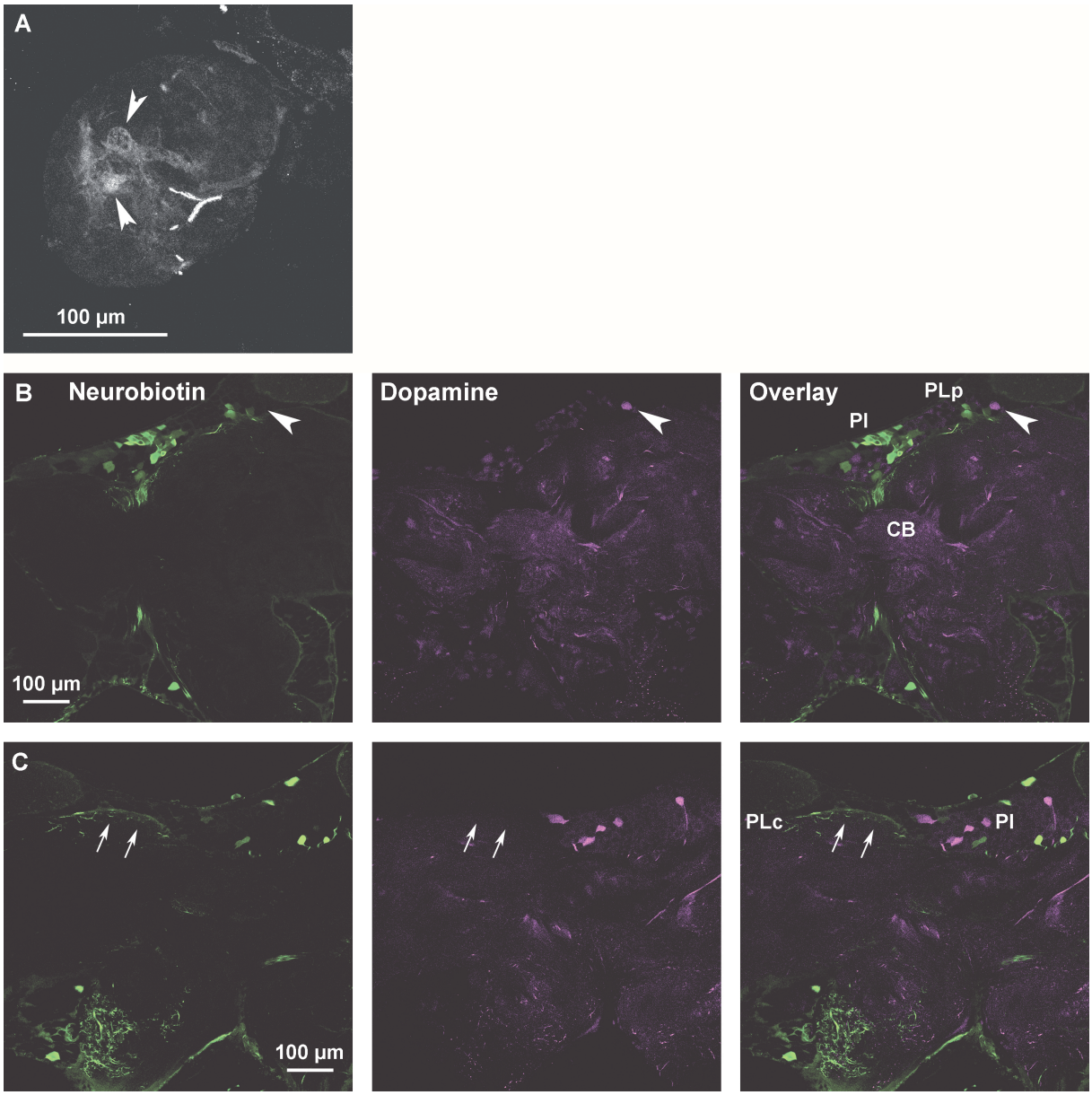

Figure 37: *Backfill of brain-to-CA projection neurons combined with dopamine immunocytochemistry*
A: Horizontal section through a corpus allatum of a female *Ch. biguttulus* after immunostaining against dopamine, maximal pixel values extracted. Dopamine immunoreactivity can be detected in cells (arrowheads) and fibers in the CA. **B and C:** Horizontal section (50 μm) through the brain of a female *Ch. biguttulus* after backill of the right corpus allatum with neurobiotin (green) and immunocytochemical staining against dopamine (purple). **B:** In the proximal pars lateralis (PLp), a dopamine immunopositive soma was found (arrowhead). Dopamine immunoreactive neurons projecting to the CA could not be detected. **C:** Arrows point to a projection to the contralateral pars lateralis (PLc), already described in other backfill experiments (see above). Positive dopamine immunostaining was not detected in this branch. PI = pars intercerebralis, CB = central body.

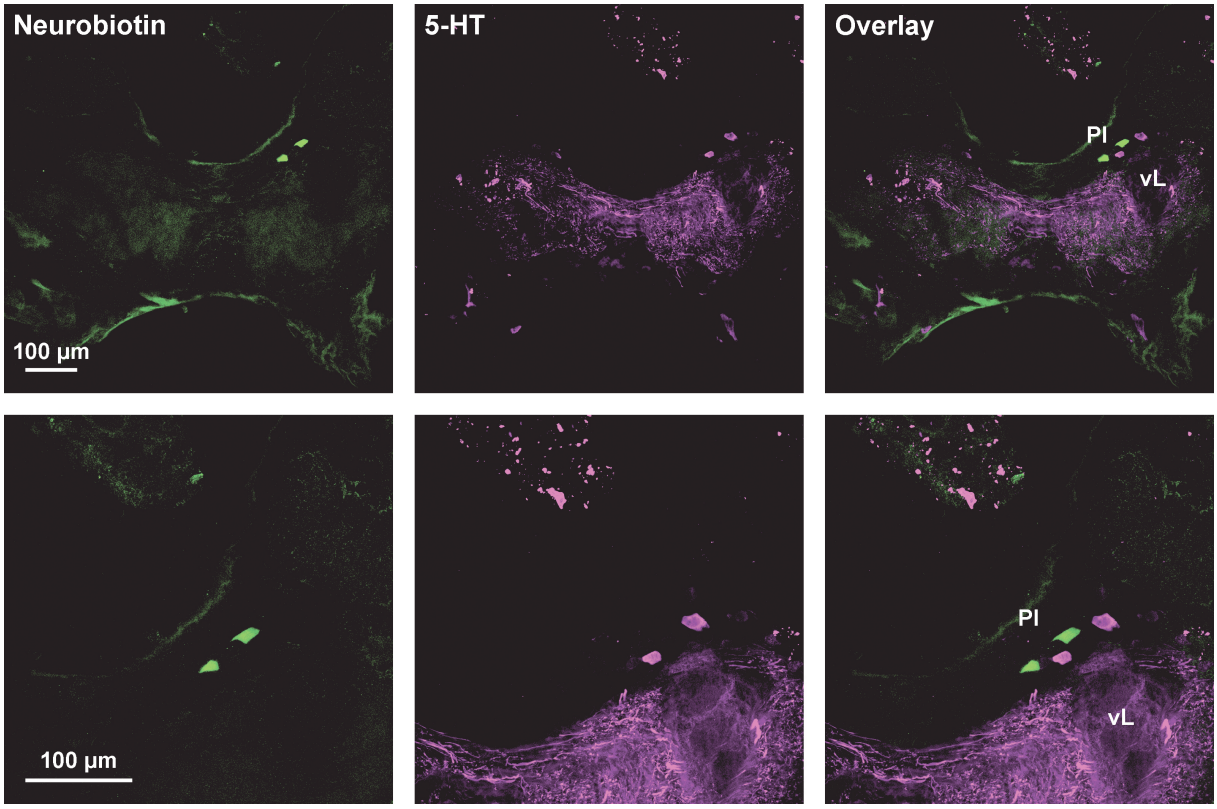

Figure 38: *Backfill of brain-to-CA projection neurons combined with serotonin immunocytochemistry*
Horizontal section (50 μm) through the brain of a female *Ch. biguttulus* after backfill of the right corpus allatum and subsequent immunostaining against serotonin. Two neurons in the lateral pars intercerebralis (PI) were immunopositive for serotonin (5-HT) and were located in the same layer as the neurobiotin traced somata. But neurobiotin and 5-HT immunoreactivity were not colocalized. The lower row shows a magnification. vL = ventral lobe.

4.4 Discussion

The CA of female *Ch. biguttulus* contain both, sources and targets, of nitric oxide release. While fixation insensitive NADPHd activity has been detected in the CA already in other species (see 4.1), it has not been possible to stain the enzyme NOS by immunocytochemical techniques (Skinner et al., 2000) in order to confirm the specificity of NADPHdiaphorase staining. In this study, the antibody against uNOS led only to very faint staining within the CA although intensive staining with the same antibody was achieved in the brain of *Ch. biguttulus* in this and earlier studies (Wenzel et al., 2005; Weinrich et al., 2008). Skinner et al. (2000) demonstrated NOS immunoreactivity only in the NCA I but not in the CA (in contrast to NADPHd activity) and explained this discrepancy with fixation insensitive enzymes other than NOS belonging to the JH synthesizing pathway that possess NADPHd activity.

One enzymatic step in JH synthesis possibly requiring NADPH or $NADP^+$ may be the conversion of farnesol to farnesoic acid. However, different studies concerning this enzymatic step provided contradictory results. Sperry and Sen (2001) demonstrated that the conversion is mediated by an alcohol oxidase in *Manduca sexta* and neither NADH nor NADPH were detectable in CA homogenates. In contrast, Mayoral et al. (2009) could show that the enzyme farnesol dehydrogenase is $NADP^+$ dependent in *Aedes aegypti*. In orthopteran species the last step in JH III synthesis is the epoxidation of methyl farnesoate, catalyzed by the methyl farnesoate epoxidase, a cytochrome P-450 reductase (Bede et al., 2001; Alzahrani, 2009) with NADPHd activity (Hammock, 1975; Feyereisen et al., 1981; Helvig et al., 2004). The reductase domain of vertebrate nNOS shows only 36% identity to cytochrome P-450 reductase (Bredt et al., 1991b) and the electron transfer mechanisms of the two enzymes are different (Knight and Scrutton, 2002). NOS unrelated NADPHd activity in the nervous system can be reduced by tissue fixation which enables the correlation of diaphorase reaction products to NOS expression in vertebrates (Matsumoto et al., 1993) as well as invertebrates (Ott and Elphick, 2003). In the vertebrate nervous system, most of NOS unrelated NADPHd activity is believed to derive from cytochrome P-450 reductases (Kemp et al., 1988). If cytochrome P-450 reductases

are fixation sensitive it is unlikely that NADPHd activity in fixated CA tissue is derived from methyl farnesoate epoxidase.

Another explanation for the absence of NOS immunoreactivity in the CA was given by Ott and Elphick (2003). They reported about a poorer sensitivity of uNOS immunostaining in insect nervous tissue in comparison to the NADPHd precipitation assay which leads to a weaker resolution. However, they also suspected that the NADPHd precipitation assay can lead to false positive results in some cases and may therefore be less specific.

Staining against citrulline provided a different tool to detect NO production sites, and it was used to clarify, whether sources of NO exist in the CA. The antibody, developed by Martinelli et al. (2002), detects citrulline with a high specificity, not only in vertebrate but also in insect nervous tissue (Siegl et al., 2009). In vertebrates, NO synthesis is the only source of citrulline production in the nervous tissue. Its alternative formation from ornithine within the urea cycle does not take place in the brain due to the absence of ornithine transcarbamylase and carbamoylphosphate synthase I (Wiesinger, 2001). This is probably also true for the invertebrate brain. In molluscs, ornithine was only detected in the hemolymph but not in the nervous tissue (Moroz et al., 1999).

Citrulline was detected in many parenchymal CA cells as described for NADPHd activity in the CA of other species like *D. melanogaster*, *D. punctata*, *Periplaneta americana*, *Acheta domesticus*, and *Leucania loreyi* (Chiang et al., 2000). Processes of these cells innervated the CC and few of them even projected into the brain. It is known that somata in the CC project to the CA (Copenhaver and Truman, 1986), but projections in the other direction, like those found in this study, have never been described before.

For nervous tissue, it was already shown that injection of the NOS inhibitor aminoguanidine (AG) into the hemolymph inhibits NO formation and reduces the accumulation of citrulline (Weinrich et al., 2008). AG was applied in the same way in this study and a similar AG mediated reduction of citrulline immunoreactivity was observed in the CA. Therefore, presence of NADPH diaphorase activity, presence of citrulline and suppression of citrulline accumulation through NOS inhibition confirm the presence of

NOS as NO source in the CA. Detection of anti-uNOS immunoreactivity has been problematic in other studies, too, (Ott and Elphick, 2002; Seidel and Bicker, 2002) and may depend on the tissue studied and subtypes or modifications of NOS enzymes contained therein.

Cellular targets for NO in the CA were also identified. By incubating the tissue with a NO donor and a phosphodiesterase inhibitor, varicose fibers were found in the CA that had accumulated cGMP. For the first time, it could be shown that peptidergic brain neurons innervating the CA are targets of NO produced by cells in the CA. Somata of those brain neurons were located in the contralateral and ipsilateral pars lateralis and the pars intercerebralis. cGMP immunopositive fibers entered the CA exclusively via the NCA I, indicating that NO release from CA cells targets brain neurons and not subesophageal neurons, entering the CA via the NCA II.

Although, nitric oxide producing neurons can be found in the same brain regions where CA projection neurons are located, NO production in the CA is not brain derived. Instead, CA cells themselves produce NO and project processes into the corpora cardiaca, some extend even further into the brain. Processes of CA cells were described before (Johnson et al., 1985), but it was never shown that CA cells project out of the CA. The findings of this study should change the picture of the CA as a simple responder organ. Obviously, CA cells give feedback in form of NO to their regulating brain neurons.

JH synthesis in the CA is controlled by allatotropins and allatostatins (Gilbert et al., 2000), but their detection rises some difficulties. The only known allatotropin is *Manduca sexta* allatotropin, sequenced by Kataoka et al. (1989). This peptide can be labeled with an antibody developed by Veenstra and Hagedorn (1993) and it has been detected in other insect species, including *Schistocerca gregaria* (Homberg et al., 2004) (see also 2.3.3).

The primary structure of allatostatin 7 was first identified in the cockroach *Diploptera punctata* (Woodhead et al., 1989; Pratt et al., 1989) as allatostatin 1. Later, it was renamed to allatostatin 7 according to its position in the A-ASTs precursor protein (Donley et al., 1993). The antibody developed in 1992 showed very high sensitivity for allatostatin 7 in comparison to other allatostatins known from *Diploptera punctata* at that time (Stay

et al., 1992).

Using the α-Dip-Ast-7 antibody in *Diploptera punctata*, the authors reported strong immunoreactivity in fibers of the NCC II entering the CC and CA (Stay et al., 1992). To control, whether CA backfills reliably label brain-to-CA projection neurons, the preparation was performed in female *D. punctata* with a subsequent staining of allatostatin. For this species, it is already known that allatostatin immunoreactive brain neurons located in the pars intercerebralis and pars lateralis project into the CA (Stay and Tobe, 2007). As expected, the CA backfill resulted in staining of allatostatin immunoreactive neurons at the described sites. Performing the same procedure with female *Ch. biguttulus* led to a different result. In this species, allatostatin expressing brain neurons do not contact the CA and their somata are not even located in the same layer as brain neurons projecting to the CA. If the molecule detected with the Dip-Ast-7 antibody in *Ch. biguttulus* brains is involved in JH production control, only indirect effects come into question. Concluding from CA backfill experiments in *D. punctata*, in this species, too, many of the brain neurons containing allatostatin do not directly project to the CA and are regarded as interneurons which indirectly control JH synthesis (Stay and Tobe, 2007). Dip-Ast-7 belongs to A-ASTs. In orthopteran species, A-ASTs could suppress JH release only in *Gryllus bimaculatus* and *Acheta domesticus* but not in *Schistocerca gregaria* or *Locusta migratoria* (Stay and Tobe, 2007, reviewed). This puts in question their allatostatic action in *Ch. biguttulus* and has to be investigated in further studies.

In case of allatotropin, the picture is different. It was already shown before that Mas-AT has no JH stimulating effect in orthopterans (Kataoka et al., 1989; Li et al., 2005). In *Ch. biguttulus*, Mas-AT immunoreactivity was neither present in the CA nor in neurons projecting from the brain to the CA. Nevertheless, the antibody did detect cells and fibers in the brain of *Ch. biguttulus*, a result that was previously described for *Schistocerca gregaria* (Homberg et al., 2004). Here, the specificity of the antibody was tested in a preadsorption assay. Immunoreactivity was almost completely abolished by applying Mas-AT, indicating that the antibody detects a highly similar peptide in *S. gregaria*.

Peptides of the same family as Mas-AT probably serve other purposes in orthopter-

ans than regulating JH synthesis. Mas-AT is a member of a peptide family with diverse actions in different insect species. Although, the family is named 'allatotropins', its members are involved in heart regulation, muscle contraction and gut movement, reviewed by Homberg et al. (2004). Also in case of allatostatins, the inhibition of JH production is most likely not the original function (Hoffmann et al., 1999). Some insects express peptides, clearly belonging to one of the allatostatin families, which do not inhibit JH synthesis in these species (Stay and Tobe, 2007). Here, other substances might fulfill the task to regulate JH production.

Several other peptides and biogenic amines like proctolin (Clark et al., 2006, *Locusta migratoria* positive, *Diploptera punctata* negative), CCAP (Dircksen et al., 1991, *Locusta migratoria*), serotonin, FMRFamide, leucokinin 1 (Helle et al., 1995, *Gryllus bimaculatus*), dopamine receptors (Granger et al., 2000, *Manduca sexta*), octopamine (Kaatz et al., 1994, *Apis mellifera*) have been detected in the CA of insects. The shape of the cGMP positive fibers indicated they were peptidergic or aminergic. The aim of the experiments discussed in the following part was to find the main neurotransmitter of the cells projecting to the CA and reacting to NO with cGMP accumulation. While CCAP and dopamine were detected in the CA of *Ch. biguttulus* females, these substances were not colocalized with cGMP. A staining with an antibody against FMRFamide after backfill of the CA and NO stimulated cGMP activation stained brain neurons in the pars intercerebralis and lateralis that project to the CA, react to NO and are FMRFamide immunoreactive. In the CA, the majority of cGMP positive fibers was also immunopositive for FMRFamide.

The peptide FMRFamide has the amino acid sequence Phe-Met-Arg-Phe-NH_2 and was discovered by Price and Greenberg (1977) in the venus clam *Macrocallista nimbosa*. FMRFamide belongs to the RFamide peptide family that occurs in invertebrates as well as in vertebrates and already in simple metazoans like cnidaria, reviewed by Cazzamali and Grimmelikhuijzen (2002). All RFamide-related peptides show a Arg-Phe-NH_2 motif at their C-terminus, reviewed by Chartrel et al. (2002). The antibody used in this study has already been applied in the sphinx moth, *Manduca sexta* (Homberg et al., 1990; Schachtner et al., 2004) and detects RFamide related peptides (Schachtner et al., 2004).

The actual peptide FMRFamide has only been detected in molluscs and annelids, other invertebrate phyla including insects were found to express related peptides with a RFamide C-terminus (Walker et al., 2009), therefore, neurons that were detected by the FMRFamide antiserum are referred to as RFamide expressing in the following.

Receptors of RFamides are metabotropic and also ligand-gated ion channels (Cazzamali and Grimmelikhuijzen, 2002). As a transmitter, RFamides can act in various ways and can have excitatory as well as inhibitory effects that are, in addition, dependent on the presence of other neuromodulators, reviewed by Kobayashi and Muneoka (1989). There are examples, where NO can interfere with the action of RFamides. At the neuromuscular junction of the nematode *Ascaris suum*, NOS activity seemed to be necessary to mediate the inhibitory effect of FMRFamides (Bowman et al., 1995), and stainings in the central nervous system of *Helix lucorum* detected RFamide positive neurons that contained NOS or were in diffusion range of NO producing neurons (Röszer et al., 2004). With synthetic FMRFamide, the production of NO could be stimulated in *Helix lucorum* (Röszer et al., 2004, 2006). In addition, RFamides may be involved in the regulation of JH synthesis. Sevala et al. (1993) found changes in FMRFamide-like peptide titer during the oviposition cycle of female *Locusta migratoria* and Stay et al. (2003) found a promoting effect of FIRFamide on JH release in female *D. punctata* but only at the end of vitellogenesis.

Concluding from the above mentioned studies and the new immunocytochemical results from this study, it can be assumed, that a RFamide peptide contributes to the control of JH release from the CA and leads to NO synthesis of CA cells acting as a feedback signal.

5 JH III titer measurements

5.1 Introduction

Different receptive stages of adult female insects are accompanied by changes in the hemolymph's JH III titer or JH III synthesizing activity of the CA (Huang et al., 1991; Hartmann et al., 1994; Schal et al., 1997). *Ch. biguttulus* females were found to start stridulating six days after imaginal molt and displayed the highest mating activity around the seventh day after imaginal molt (see chapter 3.1). As described for other grasshopper species, females are not receptive and refuse mating immediately after the imaginal molt and shortly after mating. To examine, whether changes in the female's receptivity may correspond to differences in CA activity, the JH III titer in the hemolymph was determined.

The JH III titer in young females immediately after imaginal molt, one week old females around the time of first stridulation, and in two weeks old virgin and mated females was measured by a radioimmunoassay developed by Hunnicutt et al. (1989). In addition, the juvenile hormone titer of females treated with the NOS inhibitor aminoguanidine (AG) was determined. Liquid chromatography-mass spectrometry (LC-MS) provides another tool to measure the JH III titer of individual females directly (Westerlund and Hoffmann, 2004). Both methods appear to be equally accurate, if the hemolymph samples for RIA are hexane extracted (Chen et al., 2007). To verify the results obtained by the RIA, LC-MS was applied on samples of 0-1 day old females, 5-6 day old females, and 13-15 days old virgin and mated females. Additionally, the JH III titers of female *Ch. biguttulus* in the last larval instar were measured. To explore the effect of interferences with the NO/cGMP system, the JH III titers in 13-15 day old *Ch. biguttulus* females were measured after treatment with AG and with the NO donor sodiumnitroprusside (SNP). Similar to *Ch. biguttulus* (see chapter 4.3.1), the cockroach *Diploptera punctata* has been demonstrated to express NADPHdiaphorase activity in the CA (Chiang et al., 2000; Skinner et al., 2000), suggesting that NO signaling may contribute to the control of CA functions. For comparison, *D. punctata* were also treated with AG to explore

potential effects of NOS inhibition on JH hemolymph titers.

5.2 Radioimmunoassay against JH III

5.2.1 Methods

Animals

Studies were performed with male and female grasshoppers of the species *Chorthippus biguttulus*. Females were reared from eggs that had been collected during the previous summer in the vicinity of Göttingen. Eggs were kept at 4°C for at least four months. Hatching occurred after approximately one week at 26°C and the nymphs were fed with grass and supplemental food for crickets (Nekton, Pforzheim, Germany) ad libitum. Male grasshoppers originated partly from wild populations in the vicinity of Göttingen and were partly reared in the lab from eggs. After molting into the fourth nymphal instar, experimental females were selected and separated from males. The temperature in the breeding room was 20-26°C. After the imaginal molt, female grasshoppers were individually marked with acrylic colors (Lukas CRYL TERTIA).

Females used for the titer measurements by RIA, were selected according to age, reproductive status and pharmacological treatment, see also Fig. 39:

Table 3: *Animal groups used for RIA determination of JH III titers*

Exp. group	**Age in days after imaginal molt**	**Reproductive status**	**Pharmacological treatment**
1	1-2	virgin	-
2	6-7	virgin	-
3, "Mated Non-Singers"	14-15	mated	-

Continued on next page

Table3 – continued from previous page

Exp. group	**Age in days after imaginal molt**	**Reproductive status**	**Pharmacological treatment**
4, "Singers"	14-15	virgin females that answered to a male calling song	-
5, "Non-Singers"	14-15	virgin females that did not answer to a male calling song	-
6, "AG"	14-15	virgin	injected with aminoguanidine dissolved in saline
7, "Saline"	14-15	virgin	injected with saline

Hemolymph extraction and radioimmunoassay

One day before hemolymph extraction, females of groups 3, 4, and 5 were stimulated with a recording of a male calling song to assess their responsiveness. Fig. 39 illustrates the sorting of females into experimental groups. Some of the females that responded to the

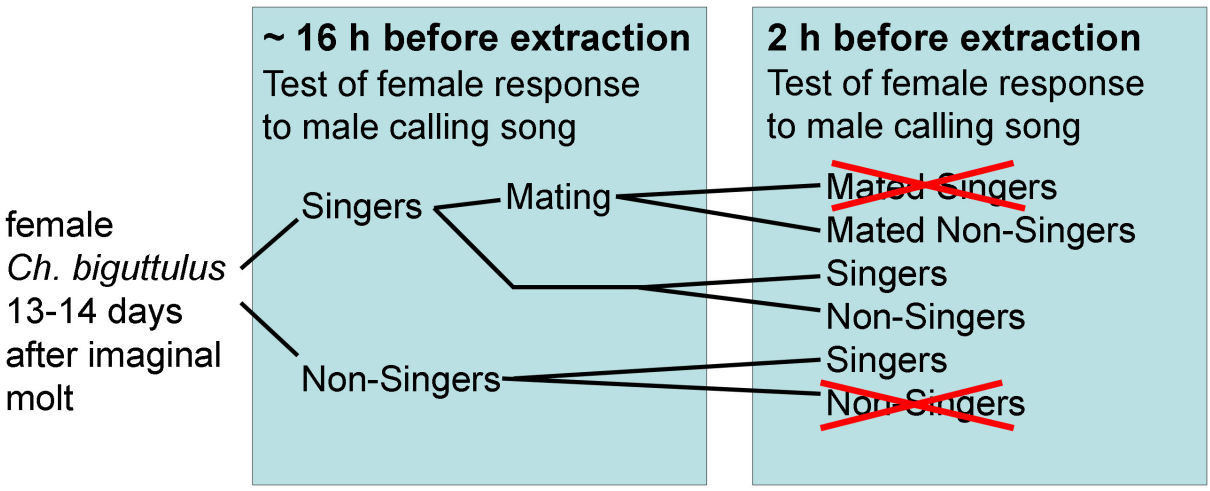

Figure 39: *Experimental animals*
One day before hemolymph extraction, the responsiveness of 13-14 days old females to a record of a male calling song was tested to distinguish responding "Singers" and not responding "Non-Singers". Some of the singers were mated on this day. On the next day, the responsiveness was tested again as a control for mating success and to exclude animals that had never answered the record. The responsiveness of females, 1-2 days after imaginal molt, and females, 6-7 days after imaginal molt, was not tested. Some animals, 14-15 days after imaginal molt, were injected with aminoguanidine without regarding their responsiveness.

acoustic stimulus ("Singers") were allowed to copulate with a male. All females tested on the first day were again tested on the following day. Mated singers that responded to the male calling song on the second day were excluded from the analysis. Song production one day after copulation may result from incomplete mating. Virgin females that did not respond to male calling songs in at least one of the stimulation periods were also excluded from further analysis. In the end, hemolymph was extracted for analysis of JH content from virgin females that were responsive ("Singers") or not responsive ("Non-Singers") to male calling song immediately before hemolymph extraction and from females that performed completed mating on the day before ("Mated Non-Singers"). Animals of group 6. and 7. were not tested and injected either with 10^{-2} M aminoguanidine in Locusta saline ("AG") or with Locusta saline ("Saline"), 4 hours before hemolymph extraction. All females were of similar age and were housed under the same conditions.

For the extraction of hemolymph, one hindleg of an animal was cut at the middle and the hemolymph was collected with a capillary pipette (DURAN), plugged to a mechanical vacuum pump (Glasfirn GmbH Giessen) with a microelectrode holder (WPI Instruments). Under slight pressure of the animal's abdomen, the hemolymph leaked from the injured leg and was collected with the pipette. Each animal provided 1-5 μL hemolymph that was directly transferred to a vial containing 500 μL acetonitrile (SIGMA-ALDRICH). By the addition of locusta saline, the solution was brought to a final volume of 1.5 mL. The vials were stored at -20°C until further processing.

To extract the lipophilic JH III from the solution, 1 mL hexane (ALDRICH) was added and the vials were vigorously vortexed. After settling down, three phases developed, with hexane as the uppermost one. In the first round, 600 μL hexane were transferred into a fresh vial, then the procedure was repeated with the remaining solution and another 800 μL were taken. The hexane was evaporated in an exsiccator (Glaswerk Wertheim) and the vials were filled with 2 mL methanol (MERCK) each.

The next steps were performed in the lab of David Borst (University of Central Florida). For the radioimmunoassay (RIA), the following solutions had to be prepared: Gel-PBST (0.05 M monophasic sodium phosphate, 0.9% NaCl, 0.1% gelatine, 0.01%

Triton X-100 in aqua dest., pH 7.3), PBS (0.6899 g monophasic sodium phosphate, 0.9 g NaCl in 100 mL aqua dest., pH 7.3), dextran buffer (250 mg dextran, 28 mg EDTA, 9.8 mg NaAzide in 50 mL PBS) and dextran coated charcoal (1 g neutralized charcoal, 10 mL dextran buffer, 240 mL aqua dest.). The principle of the RIA is based on the competition between JH III from the sample and radiolabled JH III for binding to an antibody directed against the natural 10R-enantiomer of JH III. The more JH III the sample contains, the less radiolabled JH III can bind to the antibody. After binding, the excess unbound JH III is removed from the solution and the radioactivity in the sample is measured. The following solutions were kept on ice. Stock solutions from [^3H]JH III from DuPont/NEN, 4,000 DPM/100 μL, and from a chiral specific antiserum to 10R-JH III (Hunnicutt et al., 1989), 1:8000, were prepared with gel-PBST. A JH III standard of 10R-JH III (Sigma), 5 μg/mL in methanol, was used to obtain standard values for concentrations of 5000, 1500, 500, 150, 50, 15, 5 an 1.5 ng/mL JH III. In addition total bound (TB) standards containing no JH III were prepared.

The methanol from the sample vials was evaporated in a vacuum centrifuge to collect the material at the bottom. The solid remainder was resuspended in 30 μL methanol. The assay was conducted as a two-point-assay with two reaction tubes per sample. 100 μL of the prepared [^3H]JH III stock solution were pipetted into each reaction tube. 2 μL of each sample or standard were added and the binding reaction was started by adding 100 μL of the diluted antiserum which resulted in a final concentration of 1:16,000. After incubation at room temperature for 2 hours, the tubes were chilled on ice again. 500 μL dextran coated charcoal were added to each tube and incubated for 5 minutes to capture unbound JH III and [^3H]JH III. Then, the solutions were centrifuged for 5 min at 2000 g to spin down the charcoal. The supernatant was afterwards decanted into scintillation vials which were filled with 3 mL scintillation cocktail and disintegrations per minute (DPM) were counted two times for 5 minutes per vial.

The DPM found in each standard or sample was normalized to the DPM from TB vials to obtain the %TB. To create a standard curve, the %TB values of the JH III standards were plotted against the logarithm of the JH III concentration. Sub-

sequently, a polynomic trendline was fitted to the values at concentrations 150-1.5 ng/mL.

Statistics

In the statistical analysis, data for the pg amount of JH III per μL hemolymph were compared between singing and non singing virgin females (14-15 days old), AG and saline injected females (14-15 days old), and mated and virgin females (14-15 days old). Normality could not be assumed due to the low sample size, therefore, only nonparametric analyses were conducted. Outliers were defined by the following formula: lower extremes $< Q_1 - k(Q_3 - Q_1)$, upper extremes $> Q_1 + k(Q_3 - Q_1)$ with k = 3 and Q_1 and Q_3 as upper and lower quartiles (Sachs, 2004). Outliers can derive from accidentally high measurements of scintillations or from measuring inaccuracies during the hemolymph extraction process and were removed before any statistical calculations. The variability in the groups to be compared was tested for equality using a rank sum test with a U-test statistic. Subsequently, the medians of the groups were compared by a Mann-Whitney-U-test, as well.

In the comparison of the 1-2 days old females with 14-15 days old virgin females, the U-test was not applicable, due to the fact that the former group comprised only two samples. Here, the medians of both groups were compared with a Fisher-Pitman test (Lienert, 1962). This test was also applied in the comparison of the JH III titers in 6-7 days old virgin females with the titers of 14-15 days old virgin females. It is a nonparametric test that works with measured values instead of ranks and compares small independent groups of different sizes. The null hypothesis states that the measured values of both groups have equal medians. The alternative hypothesis is that the smaller group reaches lower values than the larger. It is assumed that all observed values from both groups build up a population of possible values. All permutations where the sum of values in the smaller group is less or equal to the observed sum of values are counted. This number is divided by the total number of possible permutations to derive the probability for the occurrence of the observed sum of values. In case of the opposite alternative hypothesis (the smaller group reaches higher values than the larger), all

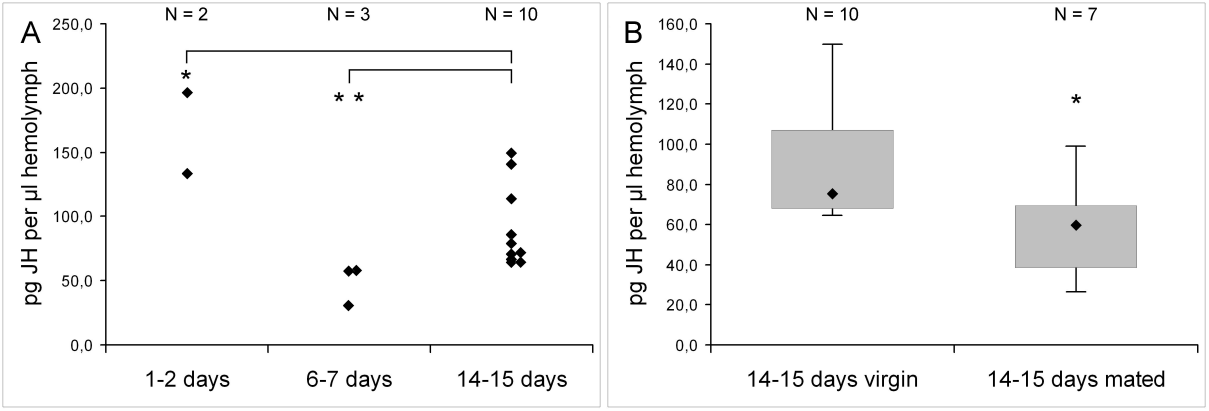

Figure 40: *JH III titer in the hemolymph at different time points after imaginal molt and in mated and virgin females*
A: The JH III titer of virgin females changes over time. 1-2 days old females have a higher JH III titer than 14-15 days old females. The JH III titer of 6-7 days old females was lower than in the older ones. Data of 1-2 days old females and 6-7 days old females were compared to those of 14-15 days old non singing virgin females with a Fisher-Pitman test. Diamonds = measured JH III titers. **B:** One day after mating, the JH III titer in the hemolymph was significantly lower than in non singing virginal females of the same age (14-15 days). The median of the measured JH III titers (diamond), the 1st and 3rd quartile (gray box), and the extreme values (whiskers) are depicted.

permutations are counted that lead to a larger or equal sum of values in the smaller group than the observed values.

5.2.2 Results

The RIA aimed to find possible natural differences in the JH III level of the hemolymph at different time points after imaginal molt and different receptivity stages. The animals used for this experiment were assigned to different groups, as described in 5.2.1 (For the experimental procedure see Fig. 39). The influence of pharmacologically suppressed NO production on the JH III level was tested with 14-15 days old females that were either injected with saline or with the NOS inhibitor aminoguanidine dissolved in saline four hours before hemolymph extraction.

The JH III titer in 1-2 days old females was compared to that of 14-15 days old virgin, non singing females with a Fisher-Pitman test. The observed sum of values in the group of the young females was 330.2 pg/μL. The number of possible permutations is 66. For this comparison, three combinations of values exist that reach a sum higher or equal than 330.2, hence, the probability for the observed sum is 0.045 and the null

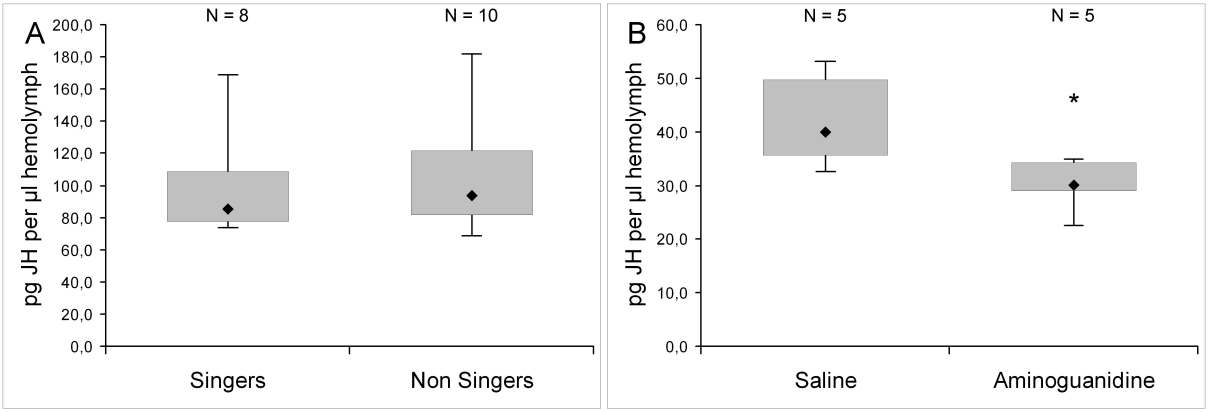

Figure 41: *JH III titer in singing and non singing virgin females and after treatment with aminoguanidine*
A: Between singing and non singing virginal females no significant difference in JH III titer was detected. **B:** Aminoguanidine treated females (14-15 days after imaginal molt) had a significantly lower JH III titer than saline injected controls, although, in previous experiments, they stridulated for longer periods upon male calling song. The median of the measured JH III titers (diamond), the 1st and 3rd quartile (gray box), and the extreme values (whiskers) are depicted.

hypothesis can be rejected. The young 1-2 days old females have a higher JH III titer than the 14-15 days old females. In the comparison of the 6-7 days old females with the 14-15 days old females, the probability was 0.0035. Those females have a lower JH III titer than the older females (Fig. 40A).

In the comparison of mated with non singing virgin females (14-15 days), seven mated females were compared to ten virgin non singing females of the same age. Unequal distributions around the median were not detected ($U_{7;10} = 31$, $N_{mated} = 7$, $N_{virgin} = 10$, $P > 0.05$) and a Mann-Whitney U-test to compare the medians of the two groups was applicable. Mated females had a median JH III titer of $\tilde{X} = 59.8$ pg/μL (interquartile range: 39.32-69.49 pg/μL), the titer of non singing virgin females was $\tilde{X} = 75.4$ pg/μL (IR: 67.77-107.04 pg/μL). The difference was statistically significant ($U_{7;10} = 14$, $N_{mated} = 7$, $N_{virgin} = 10$, $P \leq 0.025$) (Fig. 40B).

Earlier studies found that females with suppressed JH III generation do not stridulate (Loher, 1962). On account of this, it was tested if, reciprocally, silent females, have a lower JH III level in the hemolymph in comparison to singing females of the same age. In another RIA, the JH III titers in the hemolymph of 14-15 days old virgin females whose responsiveness to a male calling song had been tested shortly before hemolymph

extraction were measured. According to the behavioral test, the females were separated into eight "Singers" and ten "Non-Singers". The median JH III titer of the "Singers" was $\tilde{X}$ = 85.5 pg/μL (IR: 77.69-108.67 pg/μL), that of the "Non-Singers" $\tilde{X}$ = 93.9 pg/μL (IR: 81.60-121.53 pg/μL). Equal distributions around the median were not excluded ($U_{8;10}$ = 32, $N_{Singers}$ = 8, $N_{Non-Singers}$ = 10, P > 0.05). The subsequent Mann-Whitney U-test to compare the medians showed that JH III titers between singing and non-singing virgin females do not differ significantly ($U_{8;10}$ = 30, $N_{Singers}$ = 8, $N_{Non-Singers}$ = 10, P > 0.05, d = -8.4 pg/μL, *95%CI*: -28.4, 43.0 pg/μL) (Fig. 41A).

For the JH III titers of saline and aminoguanidine injected females equal distribution around the median was not excluded ($z(U)$ = 1.047, N_{Saline} = 5, N_{AG} = 5, P > 0.05). Saline injected females had a median JH III titer of $\tilde{X}$ = 39.9 pg/μL (IR: 35.7-49.7 pg/μL). Females that were injected with an aminoguanidine solution had a significantly lower JH III titer of $\tilde{X}$ = 30.1 pg/μL (IR: 28.9-34.2 pg/μL) ($U_{5;5}$ = 2, N_1 = 5, N_2 = 5, $P \leq 0.05$).

5.3 JH III titer measurement with liquid chromatography-mass spectrometry

5.3.1 Methods

Animals

Studies were performed with male and female grasshoppers of the species *Chorthippus biguttulus* and adult female *Diploptera punctata*. Females were reared from eggs that had been collected during the previous summer in the vicinity of Göttingen. Eggs were kept at 4°C for at least four months. Hatching occurred after approximately one week at 26°C and the nymphs were fed with grass and supplemental food for crickets (Nekton, Pforzheim, Germany) ad libitum. After molting into the fourth nymphal instar, experimental females were selected and separated from males. The temperature in the breeding room was 20-26°C. After imaginal molt, female grasshopper were individually marked with acrylic colors (Lukas CRYL TERTIA).

Cockroaches were purchased from J. Bernhardt (www.schaben-spinnen.de). Until usage, they were kept in a 55 × 35 × 27 cm box at 24°C and fed with potatoes and fruits. Female cockroaches were kept together with males and had the opportunity to mate.

Experimental groups used for the LC-MS measurement of JH III titers are summarized in table 4.

Table 4: *Animal groups used for LC-MS determination of JH III titers*

Exp. group	**Age in days after imaginal molt**	**Reproductive status**	**Pharmacological treatment**
Larvae	last nymphal instar	virgin	-
0-1 day	0-1	virgin	-

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Table4 – continued from previous page

Exp. group	**Age in days after imaginal molt**	**Reproductive status**	**Pharmacological treatment**
5-6 days	5-6	virgin	-
13-15 days virgin	13-15	virgin	-
13-15 days mated	13-15	mated	-
Aminoguanidine	13-15	virgin	injected with 30 μL Locusta saline containing 10^{-2} M aminoguanidine 4 hours before hemolymph extraction
Saline 1	13-15	virgin	injected with 30 μL Locusta saline 4 hours before hemolymph extraction
SNP	13-15	virgin	injected with 30 μL fresh SNP solution (10^{-4} M SNP in Locusta saline) 4 hours before hemolymph extraction
Saline 2	13-15	virgin	Injected with 30 μL old (72h) SNP solution (10^{-4} M SNP in Locusta saline) 4 hours before hemolymph extraction
Acetone	13-15	virgin	treated topically with 5 μL acetone 0-1 day after imaginal molt
Precocene	13-15	virgin	treated topically with 0.1 M precocene I dissolved in 5 μL acetone 0-1 day after imaginal molt
Cockroach AG	adult female *D. punctata*	mated	injected with 50 μL Locusta saline containing 10^{-2} M aminoguanidine 4 hours before hemolymph extraction
Cockroach saline	adult female *D. punctata*	mated	injected with 50 μL Locusta saline 4 hours before hemolymph extraction

Hemolymph extraction and LC – MS

Similar to the above described method (chapter 5.2.1), hemolymph was extracted by cutting a leg and collecting the fluid pouring out of it with a capillary pipette. It

was transferred to a Fisher vial containing either 100 μL methanol (MERCK) and 100 μL isooctane (MERCK) in case of the untreated females or 150 μL of each solvent when females had been injected with a solution before the extraction. From every female, 1.8 to 35 μL hemolymph were obtained. The suspension of methanol, isooctane, and hemolymph was vigorously vortexed and then allowed to settle down at room temperature for 20 minutes. To separate the phases of isooctane, proteins, and methanol, the vials were centrifuged for 20 minutes at 10,000 rpm. The upper phase, isooctane, was drawn off with a syringe (HAMILTON) and transferred to a new Fisher vial. The remains were again vigorously vortexed and left to settle down for 20 minutes at room temperature before they were again centrifuged for 20 minutes at 10,000 rpm to separate the JH III containing methanol phase from insoluble proteins. The upper phase was then transferred into the same vial as the isooctane. The isooctane phase was evaporated in the exsiccator and the remaining methanol was transferred to sample vials (ROTH) closed with a teflon/silicon septum (ROTH) that were stored for up to two months at -25°C.

Quantification of JH III in the samples by LC-MS took place at the University of Bayreuth, Dept. of Animal Ecology I, with the aid of Franziska Wende following a protocol of Westerlund and Hoffmann (2004) and Westerlund (2004).

To prepare the samples for LC-MS, they were transferred to Fisher vials and centrifuged in a speed-vac for five minutes to reach a final volume of 20 μL by the evaporation of excess methanol. The exact volume of the samples was measured before they were transferred to autosamplers (ROTH) and put into sample vials that were sealed with a teflon/silicon septum.

Separations were carried out on a Reprosil-Pur ODS3 column (Dr. Maisch GmbH, Ammerbuch), protected by a guard column (C_{18} cartridge) (Phenomenex, Aschaffenburg), at a flow rate of 0.2 mL/min with an HPLC system consisting of two pumps (LC-20AD, Shimadzu), the system controller (SCL-10A VP, Shimadzu), and an autosampler (SIL-10AD VP, Shimadzu). The methanol/water gradient changed from 30 to 100% MeOH in the first 10 minutes, stayed constant for 15 minutes at 100% MeOH and

was decreased within 1 minute to 30% MeOH again.

By electrospray ionisation (ESI) in the positive mode on a LCMS-2010A mass spectrometer (Shimadzu), the MS analysis was performed. The electrospray capillary was set to 4.5 kV, the sample cone to -5 V. The temperature of desolvation was 250°C and of the ion source 200°C with nitrogen flow rates of 4 L/min. Quantification was done by the external standards method monitoring the $[M+Na]^+$ using signal-to-noise ratios (S/N) of 3 and 10 for the limit of detection and the limit of quantification, respectively.

Hormones used in the standard were 20-hydroxyecdysone (Sigma-Aldrich), Ecdysone (Sigma-Aldrich), JH III (Fluka), JH II (SciTech), and JH I (SciTech). Standards were prepared from hemolymph of one day old adult female *Gryllus bimaculatus* and known concentrations of all five hormones.

Statistics

Before comparing the groups of interest with each other, it was tested, if normal distribution could be assumed by applying the D'Agostino Pearson omnibus test. If normal distribution could not be excluded, the Grubb's test that detects outliers in normally distributed data was applied and groups were tested for equal variances with the F-test. Depending on the F-test's result, groups were either compared with a two-tailed t-test for comparison of independent samples of equal or unequal variances, respectively. If normal distribution was excluded or the D'Agostino Pearson omnibus test was not applicable due to a low sample size, nonparametric statistics were applied. Outliers were defined as values that lie farther apart from the first and third quartile than three times the interquartile range (Sachs, 2004). These values were removed. For comparison of more than two groups, the overall H-test was used. If two groups were to be compared, they were tested for equal distribution of data around the median with a Mann-Whitney U-test. If equal distribution was not excluded, a Mann-Whitney U-test to compare the medians was applied.

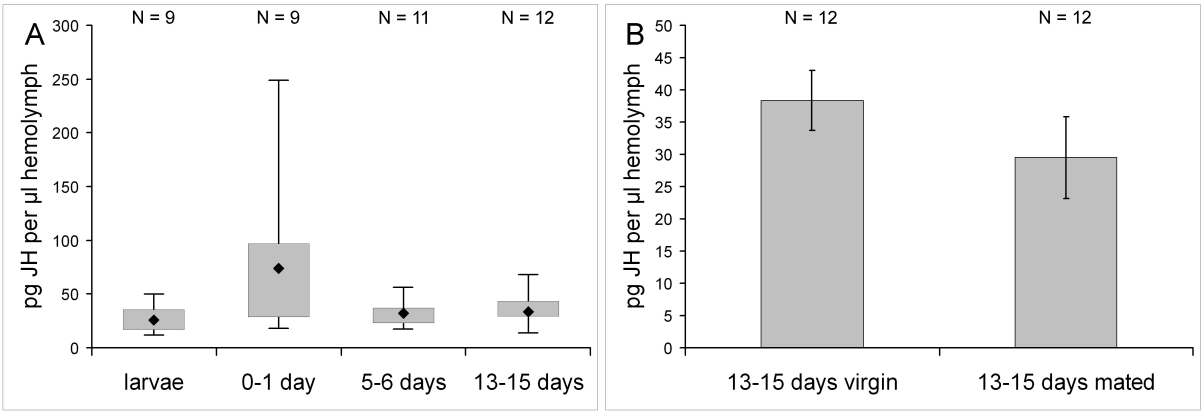

Figure 42: *LC-MS measured JH III titers at different ages and in mated and virgin females* **A:** JH III titers of virgin *Ch. biguttulus* females at different developmental stages. The highest JH III titers are measured immediately after imaginal molt (0-1 day). Until the time of first stridulation (5-6 days), the titer decreases and increases slightly at the time of first oviposition (13-15 days). 4th instar larvae (Larvae) have a much lower JH III titer than females shortly after imaginal molt. The median of the measured JH III titers (diamond), the 1st and 3rd quartile (gray box), and the extreme values (whiskers) are depicted. **B:** JH III titers of virgin 13-15 days old *Ch. biguttulus* females and 13-15 days old *Ch. biguttulus* females mated one day before hemolymph extraction. Although the JH III titer of mated females seems to be lower than in virgin females of the same age, the difference is not significant. Error bars = SEM.

5.3.2 Results

LC-MS is capable to detect JH's above 6 pg (Westerlund and Hoffmann, 2004) to 8 pg (Chen et al., 2007) and can, according to the literature, resolve quantitative differences between samples that contain more than 20 pg JH. Nevertheless, due to a highly variable baseline, it was difficult to quantify the JH content of a sample when the measured JH content of the 10 μL extract used in the LC-MS was below 100 pg. In these samples, slight changes in the definition of on- and offset of a peak in the MS readout led to large relative changes in the measured JH content. Consequently, those samples were excluded from the analysis.

JH III titers of *Ch. biguttulus* females at different ages were compared by a the nonparametric H-test, after the D'Agostino Pearson omnibus test had excluded normal distribution of the data for the 0-1 day old and 5-6 days old females ($K^2_{Larvae} = 0.45$, $K^2_{0-1day} = 9.99$, $K^2_{5-6days} = 22{,}59$, $K^2_{13-15days} = 0.60$). Due to the high variability of the values, the H-test did not detect significant differences between the groups ($H_3 = 7.24$, $P > 0.05$). Medians and interquartile ranges as well as extreme values for these groups are depicted in Fig. 42A.

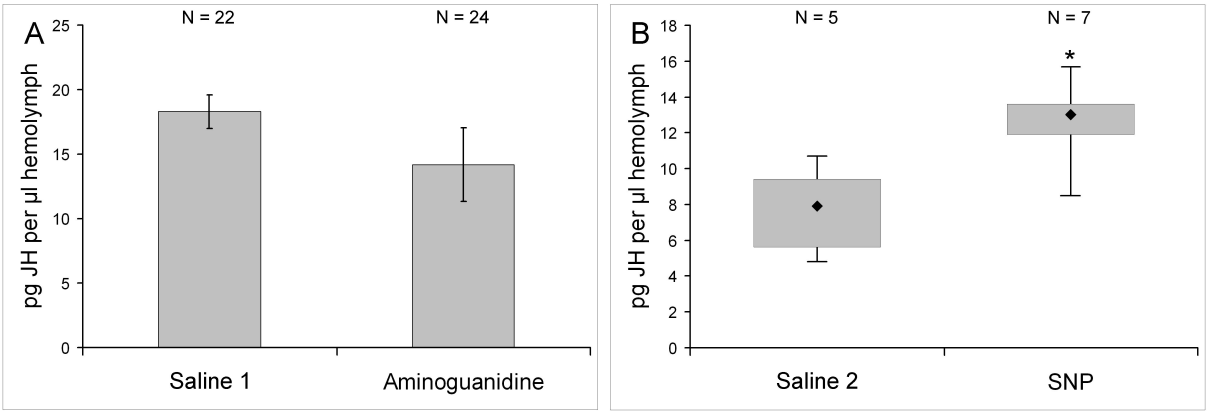

Figure 43: *LC-MS measured JH III titers in AG and SNP treated* Ch. biguttulus *females*
A: JH III titers in females injected with 30 μL Locusta saline (Saline 1) as control or 30 μL saline containing 10^{-2} M aminoguanidine (Aminoguanidine). Aminoguanidine treated females had a lower average JH III titer than their control group, but the difference was not significant. Error bars = SEM. **B:** Median JH III titers of females injected with 30 μL of a 72h old solution of Locusta saline containing 10^{-4} M SNP (Saline 2) as a control and females injected with a fresh solution of 10^{-4} M SNP in Locusta saline (SNP). The treatment increased the JH III significantly in the SNP group in comparison to the control group ($P \leq 0.01$). The median of the measured JH III titers (diamond), the 1st and 3rd quartile (gray box), and the extreme values (whiskers) are depicted.

13-15 days old virgin females had an average JH III titer of $X + SE = 38.3 + 4.68$ pg/μL, mated females of the same age had an average JH III titer of $X + SE = 29.5 + 6.31$ pg/μL (Fig. 42B). In both groups, normal distribution was not excluded (D'Agostino Pearson omnibus: $K^2_{virgin} = 0.60$, $K^2_{mated} = 5.68$) and variances in the two groups were compared with an F-test ($F_{11;11} = 0.55$, $N_{virgin} = 12$, $N_{mated} = 12$, $P = 0.33$). Since both groups appeared to have equal variances, a two-tailed t-test for independent samples with equal variances was applied, but the difference was not significant ($t_{22} = 1.13$, $P = 0.27$; confidence interval: $d = 8.8$ pg/μL, *95% CI*: -7.53, 25.25 pg/μL).

AG treated female *Ch. biguttulus* had a lower average JH III titer ($X + SE = 14.2 + 1.33$ pg/μL) than their control group that received only Locusta saline ($X + SE = 18.3 + 2.86$ pg/μL) (Fig. 43A). Since the D'Agostino Pearson omnibus test detected no deviations from normal distribution for these groups ($K^2_{AG} = 2.07$, $K^2_{Saline1} = 4.95$), parametric statistics were applied for the comparison. According to the F-test, both groups had different variances ($F_{23;21} = 0.37$, $N_{AG} = 24$, $N_{Saline1} = 22$, $P = 0.02$). The groups were compared with a two-tailed t-test for independent samples with unequal variances, but the difference in JH III titers was not significant ($t_{21} = 1.29$, $P = 0.20$; confidence

interval: d = -4.09 pg/μL, *95% CI*: -10.56, 2.47 pg/μL).

In the comparison of SNP with Saline 2 injected females (Fig. 43B), nonparametric statistics had to be applied since the sample size was too low to test for normal distribution with the D'Agostino Pearson omnibus test. Equal distributions of values around their median were not excluded according to the Mann-Whitney U-test ($U_{5;7}$ = 12.5, $N_{Saline2}$ = 5, N_{SNP} = 7) and the two groups could be compared with the Mann-Whitney U-test. The median JH III titer in the Saline 2 group was $\tilde{X}$ = 7.9 pg/μL (IR: 5.6-9.4 pg/μL) and significantly lower than that of the SNP group with $\tilde{X}$ = 13 pg/μL (IR: 11.9-13.6) ($U_{5;7}$ = 2, $P \leq 0.01$).

In order to evaluate, whether the treatment with precocene I is suitable to deplete JH III, the hormone titers of *Ch. biguttulus* females that had been treated with either 5 μL acetone or 0.1 M precocene I dissolved in 5 μL acetone immediately after their imaginal molt were measured (Fig. 44A). Hemolymph extraction took place 13-15 days after imaginal molt. The measured values in the acetone treated group were not normally distributed (D'Agostino Pearson omnibus test: $K^2_{Precocene}$ = 0.82, $K^2_{Acetone}$ = 8.01), therefore, nonparametric statistics had to be applied. Females of the acetone group had a median JH III titer of $\tilde{X}$ = 92.7 pg/μL (IR: 49.65-201.8 pg/μL), females that had been treated with precocene I had a median titer of $\tilde{X}$ = 96.15 pg/μL (IR: 54.76-181.1 pg/μL). An equal distribution of values around the median was not excluded for the two groups (Mann Whitney U-test: $U_{14;8}$ = 55, $N_{Acetone}$ = 14, $N_{Precocene}$ = 8, $P > 0.05$) and the medians were compared with the Mann Whitney U-test. A difference between the two medians was not detected ($U_{14;8}$ = 50, $P > 0.05$; confidence interval: d = -3.5 pg/μL *95% CI*: -125.6, 77.1 pg/μL).

Female cockroaches had higher JH III titers than the female grasshoppers. In saline injected cockroaches, the median JH III titer was $\tilde{X}$ = 46.6 pg/μL (IR: 33.95-82.85 pg/μL) (in comparison to a mean JH III titer of 14.2 pg/μL in saline treated grasshopper females), AG injected female *D. punctata* had a median JH III titer of $\tilde{X}$ = 30.1 pg/μL (IR: 15.75-35.28 pg/μL). In both groups, the D'Agostino Pearson omnibus test excluded a normal distribution of the data ($K^2_{cockroachS}$ = 11.09, $K^2_{cockroachAG}$ = 9.80) and their medians were

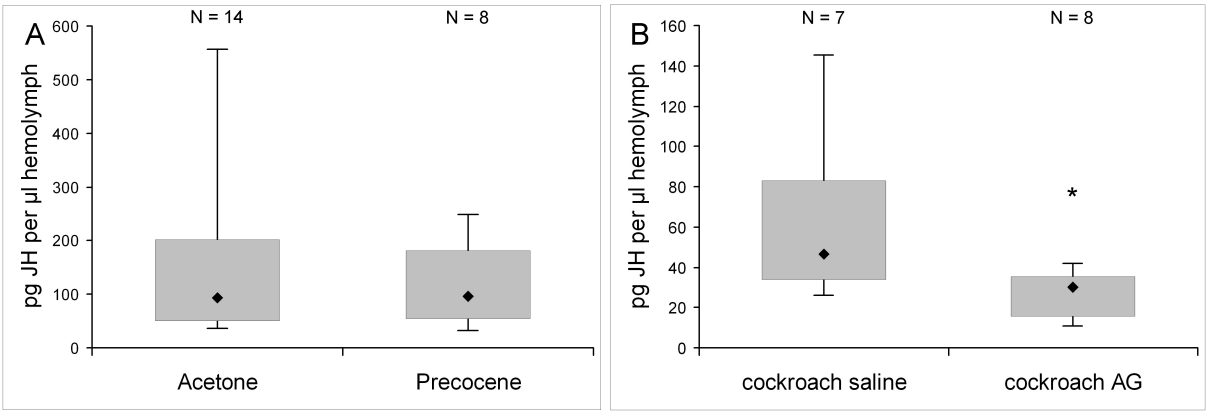

Figure 44: *LC-MS measured JH III titers in precocene I treated* Ch. biguttulus *females and AG treated* D. punctata *females*
A: To evaluate the effect of a precocene I treatment on females' JH III titer, hemomlymph samples of 13-15 days old *Ch. biguttulus* females that had been treated topically with either 5 μL acetone or with 0.1 M precocene I in 5 μL acetone immediately after imaginal molt were compared. In both groups, extraordinally high titers were measured. It is questionable, whether these values derive from JH III or from another substance possibly released through the lipid solving activity of acetone. **B:** Aminoguanidine treated *D. punctata* females (cockroach AG) had an approximately 35% lower JH III titer than females in the control group that had been injected with saline only (cockroach saline). The difference was significant ($P \leq 0.025$). The median of the measured JH III titers (diamond), the 1st and 3rd quartile (gray box), and the extreme values (whiskers) are depicted.

compared with a Mann Whitney U-test after another Mann Whitney U-test did not exclude an equal distribution of the values around their median in the two groups ($U_{7;8} = 19$, $N_{cockroachS} = 7$, $N_{cockroachAG} = 8$, $P > 0.05$). AG treated *D. punctata* females had significantly lower JH III titers than their control group ($U_{7;8} = 10$, $P \leq 0.025$) (Fig. 44B).

5.4 Discussion

The RIA is equally sensitive as LC-MS if hemolymph contents are extracted with hexane (Chen et al., 2007). But in the RIA, lipids can falsify the results by sequestering free JH III (personal communication with David Borst) and lead to overestimation of the JH III titers. This was also observed in this study. The JH III titer of virgin 14-15 days old females was approx. 90 pg/μL in the RIA, whereas the LC-MS measured a titer of approx. 38 pg/μL. Nevertheless, the relative differences in JH titer between the different behavioral states were the same, independent of the measuring method. Measured by RIA, mated females had a 20% lower JH titer than virgin females of the same age. In the

LC-MS, this difference was about 24%. Young (1-2 days old) females had a 50% higher JH III titer than 14-15 days old females in the RIA. This was also true for the LC-MS, although, the values showed large variability. AG treated females in the RIA had a 25% lower JH III titer than control animals, in the LC-MS the titers differed by 23%.

The RIA seems to be more suitable to measure the JH III titer of individual females than LC-MS when the extractable hemolymph volume and its JH III concentration are low. It requires less amounts of hemolymph and shows less variation in the measured JH III titers. For LC-MS, hemolymph was extracted with methanol/isooctane and had to reach a sample to solvent ratio of 1 : 10. Especially in young females where sometimes less than 2 μL hemolymph could be extracted, it was not possible to keep the optimal hemolymph/solvent ratio. Another difficulty arose during the JH III titer measurement of AG or saline injected females. Here, a large volume of hemolymph could be extracted, but the dilution by the additional fluid was so high that the amount of JH III in the sample vial was very low, though. Pooling the animals would be a solution to these issues but would also require much more animals. In the RIA, the JH III content of samples was determined in a two point assay. Performing more than one determination of JH III content from individual samples might also help to reduce variability in the LC-MS measured values.

One aim of the JH III titer determination was to relate behavioral states during grasshopper females' lives to changes in their hemolymph JH III titers. In a similar approach, Hartmann et al. (1994) correlated JH III synthesis rates of CA *in vitro* to different behavioral states in *Gomphocerus rufus*. They compared virgin females of defined states of sexual receptivity with females that had the opportunity to mate with males. Young *G. rufus* females in the state of 'primary rejection' had a high JH synthesis rate. With the start of 'passive readiness', the synthesis rate decreased and started to increase again when sexual receptivity increased and 'active readiness' developed. Although, *Ch. biguttulus* females show only active readiness and lack passive readiness, the time course of their JH III titers is similar to the synthesis rates measured by Hartmann et al. (1994). A similar time course of hemolymph JH III titers was also observed in the cricket *Gryllus*

bimaculatus (Westerlund, 2004) and the southwestern corn borer *Diatraea grandiosella* (Shu et al., 1997). In addition, mating led to a drop in JH III synthesis rates in *G. rufus* (Hartmann et al., 1994) and also to a drop in JH III titers in the *Ch. biguttulus* females used in this study.

It is not necessarily expectable that the JH III synthesis rate *in vitro* corresponds to the actual JH III titer in the hemolymph. The JH III titer in the hemolymph of insects is controlled by various factors. In addition to its synthesis by the CA it also depends on the presence of carrier proteins in the hemolymph and JH III esterases (Gilbert et al., 2000; Nijhout and Reed, 2008). However several studies previously showed that the JH synthesis rate is the main factor accounting for changes in the JH titer (Tobe and Stay, 1985; Feyereisen, 1985; Zhao and Zera, 2004). The results of this study, in comparison with those of Hartmann et al. (1994), indicate that hemolymph titers of JH III reflect rates of JH synthesis by the CA and vice versa.

Compared to studies on age and behavior dependent CA activity in other insect species, some differences become apparent in *Ch. biguttulus*. In *Locusta migratoria* females (Dale and Tobe, 1986), *Bombus terrestris* (Bloch et al., 2000), and *Diploptera punctata* females (Tobe et al., 1985), JH III titers are low after imaginal molt and increase with age. For several other species like moths (Cusson et al., 1999), cockroaches (Schal et al., 1997; Gadot et al., 1991) and *Drosophila* (Moshitzky et al., 1996) it was shown that mating increases JH synthesis. The results of this study, an initial decrease of JH titers in young females followed by increased titers with increased age and a reduction of JH titers after mating, emphasize that the time course of JH III titer and its effect on female reproductive behavior can vary between species.

In one part of the behavioral experiments, females were treated with precocene I. By measuring the JH III titer of those females, it was tested, whether precocene I is a suitable substance to deplete JH III in *Ch. biguttulus*. In both, the control and the treatment group, unexpectedly high measurements of the JH III titer occurred in the LC-MS. Since both groups received acetone, this effect most likely originated from the topical treatment with this solvent. Resolving precocene in acetone and applying it topically to the

abdomen is a well established method to suppress JH III synthesis in susceptible insects (Kruse-Pedersen, 1978; Okuda et al., 1996; Burns et al., 2007), and there are no reports about a JH III elevating effect of acetone. It remains questionable, if the high peaks in the LC-MS readout derive from JH III. By its fat solving side effects acetone might have released a substance with the same mass as JH III. Measuring the JH III titers of acetone treated females with the RIA could clarify this question. In the behavioral experiments (chapter 3.2), only precocene treated females showed a change in reproductive behavior, though, a little later than under natural conditions, acetone only treated control females started to stridulate and mated with males.

In both, *Ch. biguttulus* females and *D. punctata* females, inhibition of NO synthesis lowered the JH III titer. This observation suggested that NO increases the JH III. This was supported by experiments in which female *Ch. biguttulus* were treated with the NO donor SNP. SNP caused significantly elevated JH III titers. The function of NO as a messenger molecule released by the JH III producing cells in the CA has already been assumed by Chiang et al. (2000), although, they supposed an inhibitory effect of NO on JH III synthesis because of temporal shifts between the observed increases in NADPHd activity and JH III synthesis activity of the CA. But, in line with the results gained in this study, they observed an initial increase followed by a decrease in NADPHd activity in denervated CA. This pattern is also found in the JH III synthesis rate of denervated CA (Stay and Tobe, 1977). And there is an increase of NADPHd activity after mating that may come along with the increase in JH III synthesis after mating (Tobe and Stay, 1985). The discrepancies could result from the different experimental procedures. Fixation insensitive NADPHdiaphorase activity is a measure for the abundance of the nitric oxide synthase protein (Bredt et al., 1991a), but, whether NOS is enzymatically active, depends on the activation of the respective neuron. The difference between presence and activity of NOS has been demonstrated for human brain tumors (Broholm et al., 2003) but also in the brain of *Schistocerca gregaria* where only subsets of neurons that expressed NADPHd activity also accumulated citrulline, the byproduct of NO synthesis (Siegl et al., 2009). The same might be true for the CA. The elevation of NADPHd activity after mating ob-

served by Chiang et al. (2000) does not exclude a preceding increase of NO synthesizing activity that is correlated to the increase of JH synthesis.

The main target for NO in the nervous system is the soluble guanylate cyclase (Garthwaite, 2008, Review) and in the immunocytochemical stainings of this study, fibers in the CA were detected that react to NO with the accumulation of cGMP (chapter 4.3.1). Suggestions about the effect of cGMP on JH synthesis are controversial. In *Manduca sexta*, no significant effect of cyclic nucleotides could be detected (Kramer and Law, 1979). In the CA of the cockroach *D. punctata*, cGMP evoked a decrease in JH synthesis rate (Tobe, 1990), and large concentrations of cGMP were detected in CA cells of *D. punctata* when the CA were about to degrade (Chang et al., 2005). But in contrast to the present study, the mentioned studies examined the influence of 8-Br-cGMP on JH synthesis in isolated CA *in vitro*. Indirect effects of NO on the regulation of JH synthesis mediated by the brain are generally excluded in the *in vitro* assay. Since aminoguanidine and SNP were systemically applied to the grasshopper females in my studies, multiple other systems involving NO/cGMP signaling might have been affected and might have contributed to the observed effect that NO elevated the JH III titer.

6 General discussion

6.1 JH and behavior

Before interference with the females' hormonal system by allatectomy or supplemental JH, the natural reproduction related behavior was evaluated. On average six days after imaginal molt, *Ch. biguttulus* females start to stridulate and permit copulation with a male about one day after the first stridulation (see 3.1). Females can mate several times before the first egg deposition at an age of 14 to 16 days after imaginal molt. In order to correlate the hemolymph's JH III titer to the different behavioral states, JH concentrations were measured in 4th instar nymphs, in adult virgin females of 0-2, 5-7, and 13-15 days, and in mated females of 13-15 days (see chapter 5 for details). Females have a high JH III titer shortly after imaginal molt, probably due to a high JH III synthesis rate (Hartmann et al., 1994), that subsequently decreases until they reach 'active copulatory readiness'. Allatectomy experiments with precocene I showed that a high JH III titer during the first days after imaginal molt is necessary to elicit stridulation as a sign of active copulatory readiness. Chemically allatectomized females did not start to stridulate and stayed in the state of 'primary rejection'. When treated with a high dose of JH III, stridulatory behavior of allatectomized females could be restored but not their mating behavior (Fig. 45).

It can be assumed that JH III was depleted in both, allatectomized females and females in the rescue experiment, until the age when sexual receptivity starts. In a study about caste differentiation and JH titer variation, Cornette et al. (2008) applied a similar dose of synthetic JH III dissolved in 5 μL acetone to *Hodotermopsis sjostedti* termites. Following this treatment, they measured an immense increase of JH III titer from about 250 pg/μL to more than 8 ng/μL during the first 24 hours, but the additional JH III was completely degraded within seven days. If JH III is as rapidly degraded in *Ch. biguttulus*, a single dose would not be sufficient to substitute for the loss of JH III caused by allatectomy over the whole experimental period and all exogenously applied JH should have been depleted before first mating occurred.

In virgin females, a low JH III titer seems to be associated with high sexual receptivity. Immediately after imaginal molt, females have a very high JH III titer and are mute. At the lowest JH III titer level measured at an age of 5 to 7 days, females are in the state of 'active copulatory readiness' and show high mating activity. When it comes to oviposition, the JH III titer rises again, and virgin females were observed to be silent around oviposition (von Helversen, 1972; Weinrich et al., 2008). But the JH III titer does not influence female behavior in an all-or-nothing manner. After mating, females show low sexual receptivity and have a low JH III titer. As discussed in detail below, this can be explained by the effect of male accessory gland secretions eliciting secondary rejection.

The performance of stridulation probably does not only depend on the actual JH III titer but also on the maturation of the ovaries which is initiated earlier by the high JH III titer during 'primary rejection'. In many other insect species, females are unreceptive immediately after imaginal molt, and it is known that JH controls the onset of receptivity (Ringo, 1996, reviewed) by its stimulatory action on the ovaries (Loher, 1966; Sroka and Gilbert, 1971). In freshly emerged females, the ovaries are immature (Wigglesworth, 1936) and need the stimulation with JH to develop and form yolk (Chen et al., 1962). Early allatectomy prevents the maturation of the ovaries. Thus, only fully mature adult females perform stridulation to attract males and effectuate copulation. The single dose of JH III that was applied to allatectomized females 1-2 days after imaginal molt after allatectomy with precocene I was probably sufficient to enable ovary maturation and trigger the onset of reproduction promoting stridulation. But a certain JH III titer seems to be necessary to sustain the further performance of reproductive behaviors. This assumption is supported by observations in the German cockroach (Schal and Chiang, 1995). Females that were exposed to JH only during the first gonadotrophic cycle and then allatectomized were not permanently receptive and stopped to express copulatory readiness when JH was depleted.

'Secondary rejection' was of highly variable duration in the females under natural conditions. On average, it lasted between two and three days but could also persist much

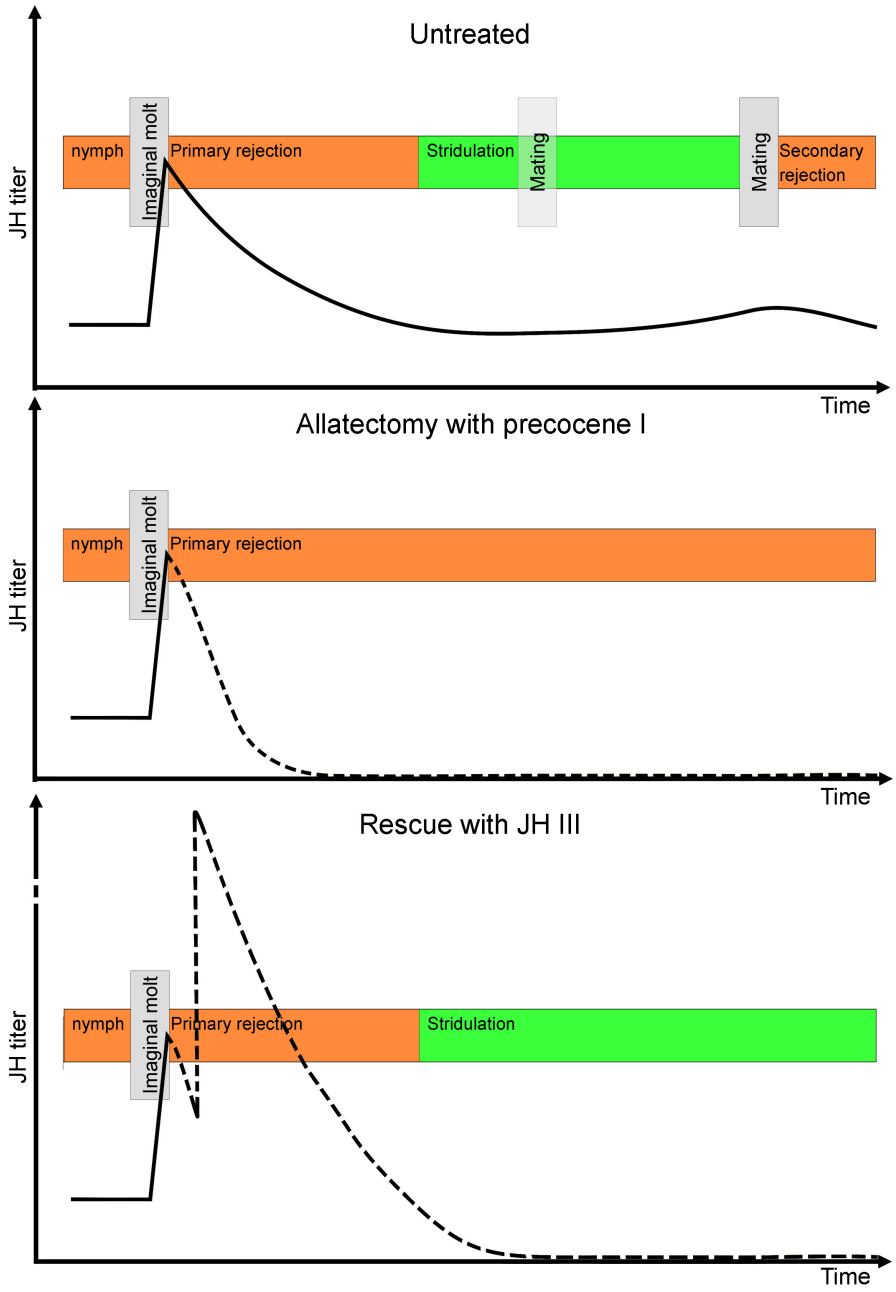

Figure 45: *Hypothetical course of JH III titers in female* Ch. biguttulus
The JH III titer was measured by RIA and LC-MS in females in the 4th larval instar, 0-2 day adult virgins, 5-7 days adult virgins, 13-15 days old virgins, and 13-15 days old mated females. Nymphs had the same JH titer as 5-7 days old adult females. 0-2 days old adult virgins had the highest JH III titer. At an age of 5-7 days, when 'active readiness' starts and mating would occur (depicted by transparent box), females had a relatively low JH titer. Older virgin females had a slightly higher JH titer. After mating, at 12-14 days of age, the JH titer was lower than in virgin females of the same age. Precocene I was delivered to the females shortly after imaginal molt to destroy the CA in an active phase of the JH III production cycle. This probably leads to a fast degradation of JH III from the hemolymph. If the allatectomy was successful, females treated in this way did not start to stridulate and remained in the state of 'primary rejection'. In a rescue experiment, allatectomized females received a high dose of JH III, which is probably completely degraded until the females reach active readiness. Females treated in this way started to stridulate but did not allow the copulation with a male.

longer. It was tested, whether the decreased JH III titer after mating is a determinant for the duration of 'secondary rejection' by applying a high dose of JH III to freshly mated females. JH III treated females showed the tendency to regain sexual receptivity earlier after mating than control females, but the difference was statistically not significant. One explanation might be the high variance in the duration of secondary rejection, but it is also possible that the JH III titer is not the most important factor that determines the onset of this behavioral state.

During copulation, the male transfers accessory gland proteins to the female which cause physiological changes in the female and also decrease sexual receptivity after mating (Hartmann and Loher, 1999; Ram and Wolfner, 2007). In other insect species like *D. melanogaster* (Moshitzky et al., 1996) or *Blattella germanica* (Schal et al., 1997), an increase (instead of a decrease) in CA activity could be observed after mating. Nevertheless, this change in JH titer seemed to play a minor role in influencing the female behavior after mating. More important factors are the tactile and chemical stimuli of the spermatophore. When virgin females of *D. melanogaster* received an application of JH III, they did not develop all typical behaviors of mated unreceptive females, reviewed by Fan et al. (1999). In case of 'secondary rejection', the alteration in JH III titer might be a mere side effect but not the ultimate cause for the occurrence of this behavioral state.

6.2 NO production in the corpora allata and its effect on JH synthesis

NADPHdiaphorase activity was observed in the CA of several insect species (*Diploptera punctata, Drosophila melanogaster, Periplaneta americana, Acheta domesticus, Leucania loreyi*) (Chiang et al., 2000) and was also present in the CA of *Ch. biguttulus*. But since Skinner et al. (2000) demonstrated a partial discrepancy between NOS immunoreactivity and NADPHd activity in the CA, it remained unclear, whether NADPHd activity is just an artefact in this tissue or truly reflects the presence of NOS. Anti citrulline stainings

performed in this study dispelled these doubts and demonstrated NO production in the CA of *Ch. biguttulus* (discussed in 4.4).

In fact, the NADPHd precipitation assay can serve as a useful marker for NOS in the CA. Chiang et al. (2000) observed an increase of NADPHd activity in the CA of mated *D. punctata* females which peaked on the 6th day after copulation. This is shortly after the JH III synthesis rate in mated females reaches its maximum (Stay et al., 1991). Chiang et al. (2000) interpret the increase in NADPHd activity as a sign for an ongoing degradation of CA tissue which eventually leads to the shrinkage of the glands and a decrease of JH III production. This hypothesis was supported by the findings that the cGMP content of CA cells rises when the glands start to shrink and that 8-Br-cGMP could reduce the JH synthesis rate in *D. punctata* (Chang et al., 2005).

In other insect species there is no or just a weak correlation between the CA size and their JH synthesis rate (Tobe and Stay, 1985; Kaatz et al., 1992; Huang et al., 1991), indicating that NO has additional functions besides degradation of the gland size and might also work as a messenger that modulates CA activity.

In this study, NO had a promoting effect on JH III titers in *Ch. biguttulus* as well as in *D. punctata*. Treatment with AG caused a decrease in JH III titers in both species and in *Ch. biguttulus* the treatment with the NO donor SNP led to an increase in JH III titer. As discussed before (see also chapter 5.4), the JH titer might not always reflect actual JH synthesis rates, and also experimental approaches in studies investigating the effect of NO on JH synthesis were different. Therefore, the decrease in JH III titer does not necessarily contradict the results of the studies mentioned above which mainly considered the JH synthesis rate of CA tissue *in vitro*. Given the fact that AG was applied systemically it might have affected also many other systems that control the JH titer of the hemolymph, such as control systems in the brain or the expression of JH esterase or binding proteins in the hemolymph.

In an earlier study about the influence of NO inhibition on female *Ch. biguttulus* reproductive behavior (Weinrich et al., 2008), virgin females were repeatedly injected with the NOS inhibitor AG. Those females answered longer and more frequently to male calling

songs than control females that had received only Locusta saline. Moreover, AG treated females even stridulated during periods close to oviposition, when sound production is normally suppressed. The effect of AG on the JH III titer might explain this result. In other insect species, the CA activity shows cyclic changes, correlated to the gonadotrophic cycle of the females (Tobe and Pratt, 1975; Chiang and Schal, 1994). The titer increases with oocyte growth and decreases following oviposition (Glinka and Wyatt, 1996; Borst et al., 2000). A similar increase of JH concentrations may be inferred from this study with *Ch. biguttulus*. If virgin females receive repeated doses of AG, their JH III titer should be permanently low enough to promote stridulation. Cyclic changes are suppressed and consequently also the cyclic changes in the stridulatory behavior of virgin females are absent.

By immunocytochemical stainings against cyclic GMP, the cellular targets of NO were detected in the CA. Fibers entering the CA via the NCA I reacted to NO with the accumulation of cGMP. They belonged to brain neurons located in the dorsal PI and PL. This suggests that NO might function as a retrograde transmitter in the CA which provides feedback from the JH producing parenchymal cells to peptidergic fibers controlling the synthesis of JH. In various other invertebrate systems, NO was already reported to work as a retrograde messenger like the fly neuromuscular junction (Wildemann and Bicker, 1999), the photoreceptor-to-monopolar cell synapse in the locust lamina (Elphick et al., 1996), and the crustacean cardiac ganglion (Scholz et al., 2002).

Most of the cGMP positive fibers in the NO stimulated CA expressed an RFamide-like peptide. Relations between NO and FMRFamide signaling were previously identified in invertebrates (*Ascaris suum* (Bowman et al., 1995) and *Helix lucorum* (Röszer et al., 2004, 2006)). In *A. suum* NOS containing cells in the hypodermis expressed receptors for FMRFamide-like peptides and in the nervous system of *H. lucorum*, synthetic FMRFamide could induce the production of NO while NADPHd positive neurons received FMRFamide positive terminals. In addition, FMRFamide-related peptides seem to play a role in the oviposition cycle of female locusts (Sevala et al., 1993) and are expressed in the same brain neurons as Mas-AT in *Manduca sexta* (Bhatt and Horodyski, 1999), suggest-

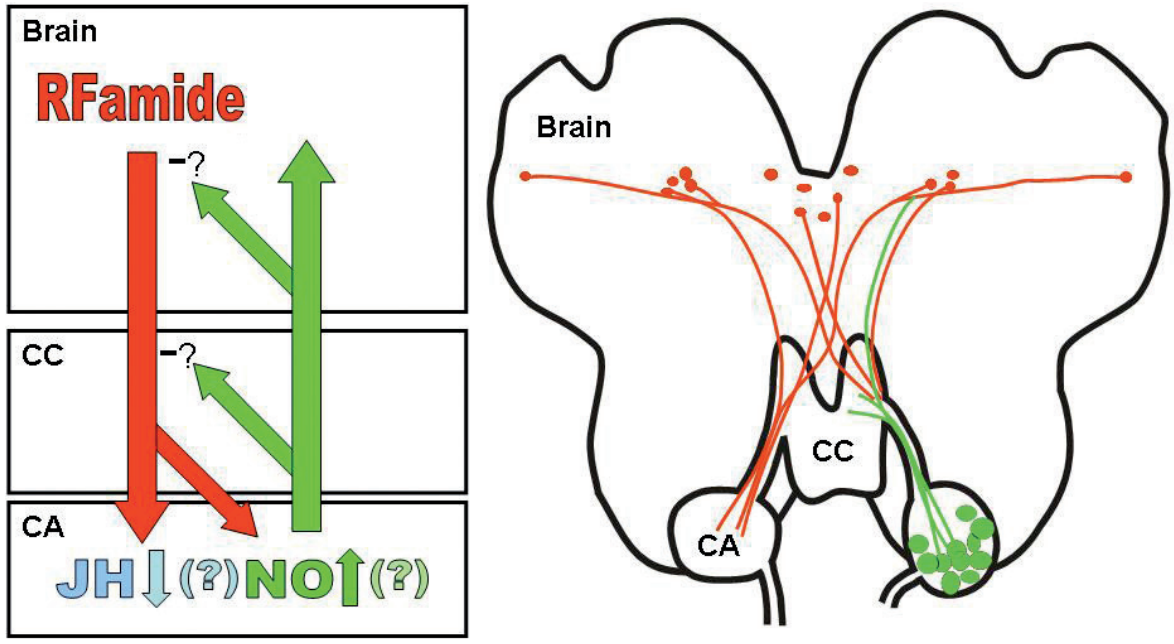

Figure 46: *Hypothetical scheme for the effect of NO as a feedback signal at the corpora allata*
Immunocytochemistry against citrulline revealed NO producing cells (green) in the corpora allata (CA) that send processes into the corpora cardiaca (CC) and the protocerebrum (brain). RFamide positive fibers in the CA accumulated cGMP in response to NO and were identified as targets of NO signaling (red). They belonged to neurons in the brain located in the pars intercerebralis and lateralis. On the left, a hypothetical scheme of the interplay between NO and RFamide and their effect on JH synthesis is depicted. RFamide probably reduces JH synthesis and elicits NO release from CA cells. Either on CA, CC, or brain level, NO leads to an inhibition of the RFamide releasing neurons and can thereby increase JH synthesis.

ing a regulating function on hormonal systems of insects. FMRFamide-related peptides do not exclusively work as neurotransmitters or neuromodulators inside the nervous system, but are also released into the hemolymph to exert hormonal functions (Sevala et al., 1993). RFamide positive neuronal terminals in the CA might therefore either release the peptide into the hemolymph or affect the CA cells in a synaptic or paracrine way.

So far, no study provides substantial evidence for an effect of RFamides on JH synthesis. It can only be speculated that the RFamide-related peptide released by fiber terminals in the CA might suppress JH synthesis in *Ch. biguttulus* since it was expressed in fibers entering the CA from the brain and it is known that the CA of *D. punctata* are under inhibitory control of the brain (Stay and Tobe, 1977; Woodhead and Stay, 1989; Lenik et al., 2009). As a consequence, the RFamide would have two functions in the CA. It

decreases JH synthesis and at the same time increases NO production. If NO acts as a feedback signal from the JH synthesizing cells, it should have an inhibitory effect on RFamide release and thereby lead to an increase in JH production. The hypothetical scheme of the action of NO and RFamide in the retrocerebral complex and on the JH synthesis is illustrated in Fig. 46.

6.3 Parallels to the vertebrate hypothalamus-hypophysis-axis

The insect retrocerebral complex is considered as an analog to the pituitary gland in vertebrates. In this comparison, the CC correspond to the posterior lobe or neurohypophysis while the CA are analogous to the anterior lobe of the vertebrate pituitary gland, the adenohypophysis (Scharrer, 1987).

Previous comparisons between the CA and the anterior pituitary were related to functional, rather than to anatomical similarities. Both, juvenile hormone secreted by the CA and the various hormones released by the adenohypophysis, are of similar importance for the animals' development and reproduction and both glands are regulated by the brain (Scharrer and Scharrer, 1944).

The corresponding brain regions that control hormone release from the CA and the anterior pituitary are the pars intercerebralis (PI) respectively the pars lateralis (PL) in insects and the hypothalamus in vertebrates (de Valesco et al., 2007). Analogy between these regulatory centers extends beyond principal functions. Based on structural similarities of peptides released by neurosecretory cells in PI and hypothalamus (Nässel, 2002) and developmental studies (Hartenstein, 2006), a common ontogenetic origin for these regions has been suggested that is supported by genetic comparisons (Tessmar-Raible et al., 2007).

The last common ancestor of protostome and deuterostome bilaterian animals lived approx. 910 million years ago (Hedges et al., 2006) and most likely had a centralized nervous system (Arendt and Nübler-Jung, 1994; Denes et al., 2007). The neurosecretory systems of bilaterian animals developed before the separation of protostomes and deuterostomes (de Valesco et al., 2004; Tessmar-Raible, 2007), and the pars intercerebalis/lateralis-

corpora cardiaca-system of insects is seen as the homolog to the hypothalamus-pituitary-axis of vertebrates (Hartenstein, 2006; Tessmar-Raible, 2007; Tessmar-Raible et al., 2007).

The adenohypophysis takes an exceptional position within the neuroendocrine system of vertebrates since it arises from a different placode than hypothalamus and neurohypophysis during embryonic development (Kawamura et al., 2002), and it is controlled by humoral factors delivered via the hypophyseal portal system (Popa and Fielding, 1930).

The literature provides numerous similarities between the CA and the adenohypophysis which will be summarized in the following. The comparison will also include the immunocytochemical results of my PhD study that support the view of a possible common origin of the insect CA and the vertebrate adenohypophysis.

Functional and positional similarities are the most apparent resemblances between adenohypophysis and CA. The neurohypophysis is seen as a homologue to the CC (Tessmar-Raible, 2007; Tessmar-Raible et al., 2007), adjacent to this organ, the adenohypophysis is located (Amar and Weiss, 2003, reviewed). In insects, the CA take the corresponding position and are either located adjacent to the CC or they are connected to the CC via the nervus corporis allati I (Cassier, 1979). The CA are not only located at a similar position as the adenohypophysis, hormones released by the adenohypophysis (e.g. luteinizing hormone or growth hormone) and JH released by the CA fulfill similar general functions in vertebrates and insects (Scharrer, 1987) that include stimulation of the vitellogenesis and the regulation of development and reproductive behavior (Hartfelder, 2000).

Jacobs and Gates (2003) argued against an insect homolog of the adenohypophysis due to the lack of the *Pit-1* gene, necessary to initiate the development of growth hormone, prolactin, and thyroid stimulating hormone secreting cells in the vertebrate adenohypophysis (Radovick et al., 1992). But insects do possess the homeobox gene *Pitx1* which activates pituitary specific promoters (Vorbruggen et al., 1997, *D. melanogaster*), (Shiomi et al., 2007, *Bombyx mori*) and interacts with Pit-1 (Szeto et al., 1996; Tremblay et al., 1998). In insects, *BmPitx* initiates the development of neurosecretory cells and the diapause hormone secreted by these cells can be detected in the CA (Shiomi et al., 2007).

The developmental origin of the CA is still under debate. While some studies found

them to originate from mesodermal structures (Tiegs and Murray, 1938, *Calandra oryzae*), (de Valesco et al., 2004, *Drosophila*) it is mostly assumed that they develop from ectoderm (*P. rapae*, *C. suppresalis*, *Bombyx mori*, *L. viridana*, *Apis mellifica*, *Dixippus morosus*, *L. migratoria*, reviewed by Kobayashi and Ando (1983), *Drosophila*, *Oncopeltus*, reviewed by Hartenstein (2006)). A detailed description of CA ontogeny is provided by Hartenstein (2006). He states an invagination from the ventro-anterior ectoderm of the maxillary segment as the origin of the CA primordium, which migrates dorsally and contacts the CC. The adenohypophysis of vertebrates is of ectodermal origin. It develops from Rathke's pouch which invaginates toward the infundibulum and makes contact to the neurohypophysis after complete separation from the oral roof ectoderm (Rizzoti and Lovell-Badge, 2005). Rathke's pouch that in vertebrate gives rise to the adenohypophysis expresses *optix/Six6*. This homoedomain gene is also expressed in the ventral-anterior ectoderm of the maxillary segment in insects (Wang et al., 2007).

The CA produce JH while the adenohypophyseal neurosecretory cells produce peptidergic hormones which seems to be a fundamental difference, but both organs contain similar peptides and proteins involved in the synthesis pathways of the signaling molecules. Adrenocorticotropic hormone (ACTH) produced by the adenohypophysis and melanocyte stimulating hormone (MSHα) produced in the intermediate lobe of the pituitary are both derivatives of the precursor protein POMC (proopiomelanocortin) (Scully and Rosenfeld, 2002). A POMC derivative, β-endorphin, is also produced in the CA (Hansen et al., 1982), and the CC contain MSH (Schoofs et al., 1987). JH is synthesized from methyl farnesoate by JH epoxidase, a cytochrome P450 enzyme named CYP15A1 (Helvig et al., 2004). CYP15A1 belongs to the CYP2 clan of cytochrome P450s (Baldwin et al., 2009). Similarly, cytochrome P450s of this clan were also found in the vertebrate adenohypophysis: CYP1A1 could be induced in gonadotrophs (Anderson et al., 1993, rainbow trout) but is also produced constitutively (Huang et al., 2000, rat). Additional, more closely related CYP2 clan members, CYP2C9 and CYP2J2 (Enayetallah et al., 2004), were identified in the adenohypophysis, although, the celltype expressing these enzymes was not identified.

The CA are innervated by the brain and the CC (Virant-Doberlet et al., 1994), whereas the adenohypophysis is assumed to be primarily regulated by humoral signals through the hypophyseal portal system (Popa and Fielding, 1930). Only in teleost fishes, the adenohypophysis is directly innervated by the hypothalamus (Peter et al., 1990), which has been interpreted as a highly derived condition (Sower, 1998). However, Liu (2004) found direct innervation of the human adenohypophysis via projections through the pituitary stalk, a result that challenges the view of an exclusive humoral control of the mammalian adenohypophysis and provides a further similarity to the insect CA.

The occurrence of RFamides in hypothalamus and PI/PL respectively in pituitary and CC is interpreted as a sign for the common phylogenetic origin of these organs (Hartenstein, 2006; Tessmar-Raible, 2007). In this study, a RFamide was immunocytochemically detected in fibers innervating the CA of *Ch. biguttulus*. Several other studies reported about FMRFamide-like peptide expressing cells that innervate insect CA (Carroll et al., 1986; Eichmüller et al., 1991; Helle et al., 1995). In *D. punctata*, FIFRamide promoted JH release from the CA (under certain conditions) (Stay et al., 2003). At the adenohypophysis, both inhibitory and excitatory effects of RFamides on hormone release are known, examples are KiSS-peptide 1 which stimulates LH release (Navarro et al., 2005) and GnIH, the gonadotropin inhibiting hormone (Tsuitsui et al., 2000). Both peptides directly affect adenohypophyseal cells. SIKPSAYLPLRFamide, was identified in 2000 and named gonadotropin-inhibitory hormone (GnIH) due to its diminishing effect on gonadotropin release (Tsuitsui et al., 2000). Besides GnIH, several other RFamide-related peptides were found to regulate the release of gonadotropins from the anterior pituitary.

In this study on *Ch. biguttulus*, the production of NO in CA cells was demonstrated. Citrulline accumulation was found in almost all cells of the CA, amongst them, cells possessing many processes and even projecting out of the glands into the CC. Similarly, the adenohypophysis contains NO generating folliculo-stellate cells wiring the whole gland (Fauquier et al., 2002). Additionally, the LH and FSH secreting gonadotropes possess the ability to produce NO (Ceccatelli et al., 1993). Until now, the CA are considered to be homogeneous tissues, only consisting of one cell type that produces JH (Johnson

et al., 1985). Whether indeed all CA cells produce JH, should be reconsidered since morphological characteristics of CA cells appeared quite variable, suggesting the existence of different cell types that probably serve diverse functions.

The demonstration of similar functions of nitric oxide (NO) in the insect PI-CA-axis controlling JH production and the vertebrate hypothalamus and adenohypophysis provides another parallel between both tissues. In the hypothalamus, nitric oxide synthase (NOS) is expressed by oxytocin- and vasopressin-producing magnocellular neurons of the paraventricular and supraoptic nuclei and is supposed to regulate the secretion of gonadotropin-releasing hormone and corticotropin-releasing hormone from parvocellular paraventricular neurons (Bernstein et al., 1998, reviewed). In the adenohypophysis, nitric oxide (NO) is, as mentioned above, produced in gonadotropes and folliculo-stellate cells (Ceccatelli et al., 1993) and seems to function as a regulator of luteinising hormone (LH) secretion (Yamada et al., 1997), prolactin (Duvilanski et al., 1995), and growth hormone release (Rubinek et al., 2005). As reviewed by Rosselli et al. (1998), it thereby influences vertebrate reproductive behavior.

Similarly, NO is involved in the regulation of insect reproductive behavior. Inhibition of NOS by systemic application of aminoguanidine increased the responsiveness of *Chorthippus biguttulus* females to male-derived reproductive signals, suggesting that NO formation suppresses reproductive behavior in grasshopper females (Weinrich et al., 2008). In addition, studies on *D. punctata* females revealed changes in NADPHdiaphorase activity in the CA associated with periods before and following mating (Chiang et al., 2000).

Immunocytochemistry and JH titer measurements performed in this thesis revealed further parallels in the control of hormone release between insect CA and vertebrate adenhypophysis. Like the gonadotropes in the anterior pituitary, the parenchymal cells in the CA produce nitric oxide. Moreover, inhibition of NO production leads to a decrease in JH titer, similar to the decrease in gonadotropin release from the vertebrate pituitary under NO deficiency (Barnes et al., 2001).

Similarities between the CA and the adenohypophysis can be found on morphological,

Table 5: *Similarities between vertebrate adenohypophysis and insect corpora allata*

	Adenohypophysis	**Corpora allata**
Hormone functions	Regulation of development, growth, and reproduction (Amar and Weiss, 2003)	Regulation of development, growth, and reproduction (Hartfelder, 2000)
Position	Adjacent to neurohypophysis (Amar and Weiss, 2003)	Adjacent or connected to CC (Cassier, 1979)
Innervation	Profound innervation from hypothalamus in teleost fishes (Peter et al., 1990), sparse innervation from hypothalamus in humans (Liu, 2004)	Direct innervation from PI/PL and CC (Virant-Doberlet et al., 1994)
Expression of *Optix/Six6*	Rathke's pouch (Wang et al., 2007)	Ventral-anterior ectoderm of the maxillary segment (Wang et al., 2007) (probably origin of CA primordium (Hartenstein, 2006))
POMC-derivatives	ACTH (Scully and Rosenfeld, 2002), β-endorphin (Amar and Weiss, 2003)	β-endorphin (Schoofs et al., 1987)
Cytochrome P450	CYP1A1 (Anderson et al., 1993; Huang et al., 2000), CYP2C9, CYP2J2 (Enayetallah et al., 2004)	CYP15A1 (Helvig et al., 2004)
RFamides	KiSS-peptide1 (Navarro et al., 2005), GnIH (Tsuitsui et al., 2000)	RFamide-like peptide (Helle et al., 1995; Stay et al., 2003, this study)
Nitric oxide production	Folliculo-stellate cells, gonadotropes (Ceccatelli et al., 1993)	All CA cells including types with many projections (this study)
Nitric oxide function	Decreased gonadotropin release under NO deficiency (Barnes et al., 2001)	Decreased JH titer under NO deficiency (this study)

physiological and most likely on the developmental level, an overview of the discussed details is given in table 5. To find more support for the hypothesis of a common evolu-

tionary origin of the two organs, the CA should be studied in more detail with regard to their fine structure and formation during embryonic development.

6.4 Further experimental approaches

The new findings about the influence of juvenile hormone on female *Ch. biguttulus* behavior and its interplay with the nitric oxide/cGMP system answered some questions but also raised new ones.

In the behavioral experiments, only synthetic JH III was used which can be degraded by juvenile hormone esterase. This study suggested that the transient elevation of JH levels resulting from a single exogenous application can rescue some (stridulation) but not all (no mating) components of reproductive behavior that are lost after JH depletion following allatectomy. JH analogs like methoprene (Riddiford and Ashburner, 1991; Wilson, 2004; Zera and Zhao, 2004) or pyriproxyfen (Zhang et al., 1998) have the same biological effect as juvenile hormone but are not as quickly degraded by endogenous enzymes. Using one of these analogs may be a possibility to test, whether performance of mating behavior may require a prolonged presence and influence of exogenously applied JH in allatectomized females. Also a lower dose of JH III or the analog might be more suitable to mimic the natural JH III titer in females.

To prove the hypothesis that repeated applications of aminoguanidine permanently lower the JH III titer in female *Ch. biguttulus*, the JH III titer of AG treated virgin females should be determined for several time points and oviposition cycles in comparison to a control group.

In contrast to observations in other insect species, the JH III titer of female *Ch. biguttulus* was lower after mating. Since it was only determined on the next day after mating, it cannot be excluded that the titer may rise but after a temporal delay. Therefore, the JH III titer of mated females should be followed over several days to document its time course after mating.

In line with the results of this study, a lower CA activity in mated grasshopper females compared with highly receptive virgin females of the same age has been observed

in mated females before (Hartmann et al., 1994). This contrasts with results from other insect species, suggesting a specialty of grasshoppers that is not shared by all insects. Alternatively, the selection of mated females may introduce some bias to the measurements, as it has been described by Schal et al. (1997). If young females are given the opportunity to mate with males, the females in the most advanced state of maturation will mate and will constitute the group of the mated females. Differences in the measured CA activity may therefore be caused by the differences in maturation and not by mating itself. In the behavioral experiments of this study, older females shortly before or after first oviposition were mated. It is known that virgin *Ch. biguttulus* females loose their receptivity around the time of oviposition (von Helversen, 1972; Weinrich et al., 2008). The JH III titer in female insects rises toward the time of oviposition and drops afterwards (Chiang and Schal, 1994; Tobe and Pratt, 1975). It could have been the case that all females which rejected copulation had higher JH titers because they were close to first oviposition while those females that mated were those that had finished their first oviposition cycle and contained lower JH titers. This concern could be addressed by mating experiments with younger females, at the beginning of their receptive phase where interference with oviposition cycles can be excluded.

Immunocytochemistry demonstrated that NO is produced by cells in the CA and stimulates cGMP accumulation in RFamide immunopositive cells entering the CA from the brain. In the brain, also Dip-Ast-7 and Mas-AT positive cells reacted to NO. This brings up the questions in which way these peptidergic neurons might react to NO and how the release of their transmitters changes the CA activity. NO induced changes in the expression level of mRNA or peptide could be evaluated by in situ hybridization or ELISA. Furthermore, synthetic FMRFamide could be administered to female *Ch. biguttulus* in order to assess its effect on JH III titer and reproductive behavior.

Stridulatory behavior in grasshoppers is controlled by the brain (Bauer and von Helversen, 1987; Hedwig and Heinrich, 1997; Heinrich et al., 2001). If the JH titer influences female stridulatory behavior, it should somehow influence neuronal function in brain regions that regulate behavior. Evidence already exists that JH does affect the nervous

tissue (Unnithan et al., 1978; Anton and Gadenne, 1999; Gadenne and Anton, 2000) and can change the levels of neurotransmitters as it was shown for dopamine in male honey bees (Harano et al., 2008). Similarly, JH could change the production and release of NO in the brain. To test, for example, whether a high JH titer inhibits female stridulatory behavior by increasing NO production in the brain, citrulline stainings could be performed after incubating the brains with JH III.

7 References

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8 Appendix

Phosphate buffer

- $NaH_2PO_4 \cdot H_2O$ (MERCK) 84mM
- KH_2PO_4 (MERCK) 16mM

PBS

- $NaH_2PO_4 \cdot H_2O$ 1.5mM
- $Na_2HPO_4 \cdot 2H_2O$ (MERCK) 8.4mM
- NaCL (MERCK) 145.3mM

Locusta saline

- NaCL 140mM
- KCl (MERCK) 10mM
- $NaH_2PO_4 \cdot 2H_2O$ (MERCK) 4mM
- Na_2HPO_4 (MERCK) 5mM
- Sucrose (MERCK) 90mM
- $CaCl_2 \cdot 2H_2O$ (MERCK) 2nM

Sørensen phosphate buffer

- Solution A: NaH_2PO_4 0.1M
- Solution B: Na_2HPO_4 0.1M

  Solution B is added to solution A until the pH reaches a value of 5.0.

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